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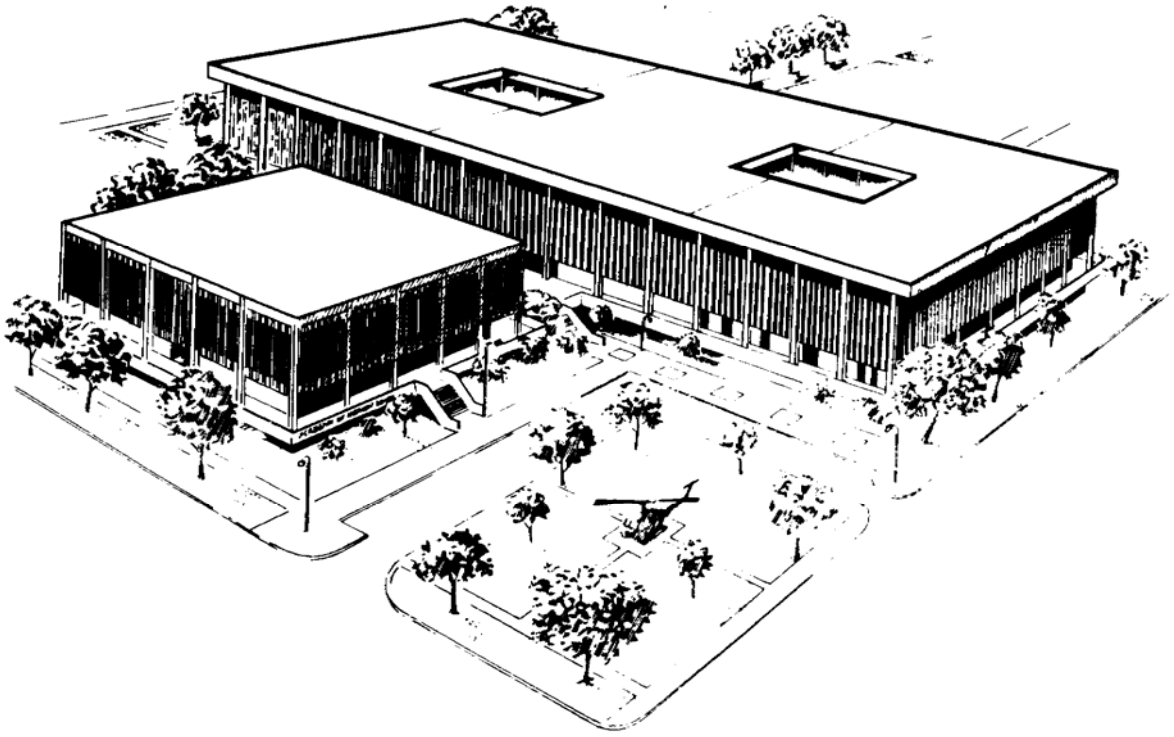
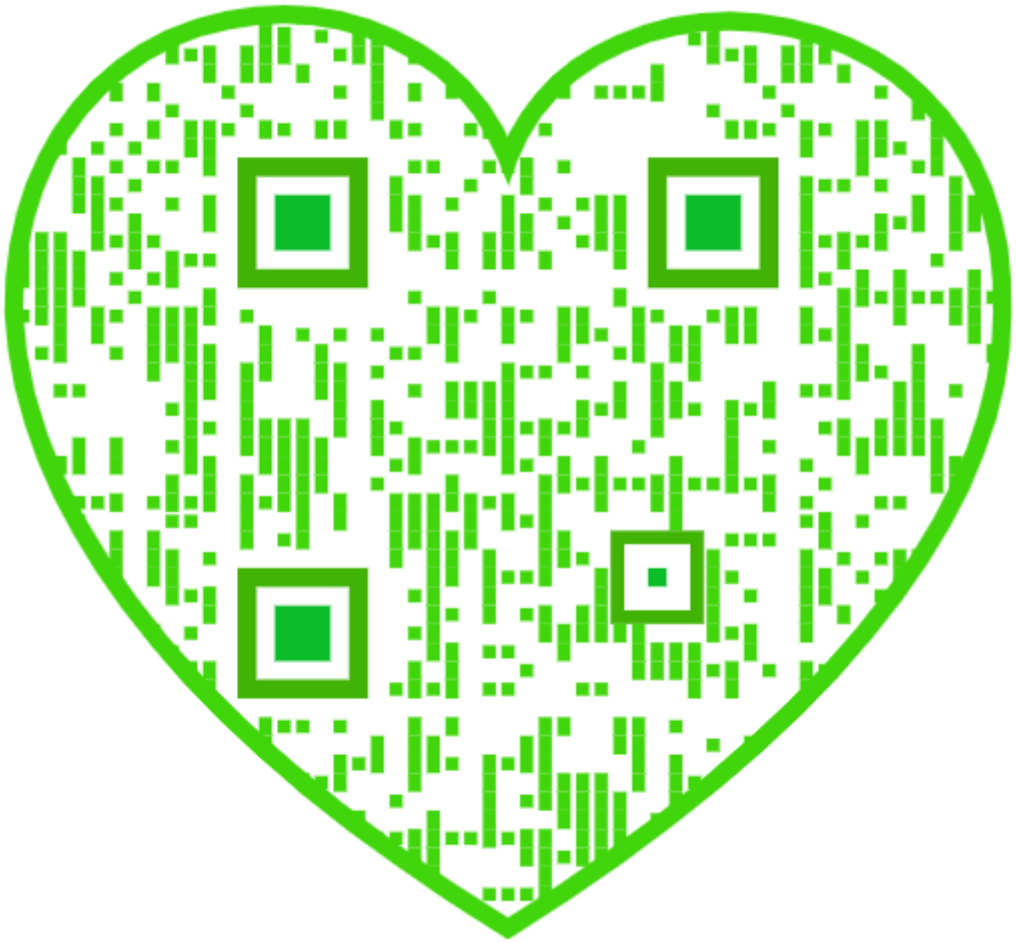


BACTERIOLOGY

SUBCOURSE MD0856 EDITION 200



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CORRESPONDENCE COURSE OF THE
U.S. ARMY MEDICAL DEPARTMENT CENTER AND SCHOOL

SUBCOURSE 856

BACTERIOLOGY

INTRODUCTION

The whole purpose of clinical laboratory procedures is to provide the clinician doing diagnostic work with specific information needed to round out his picture of the disorders he has observed in the patient. Clinical bacteriology can contribute its part by supplying data about the microscopic life involved and the susceptibility of such life to particular drugs. To identify bacterial growth, you must take certain steps that will enable you, through a process of elimination, to choose the microscopic form that fits the findings you have obtained. Steps that are often essential include:

1. Observing the type of growth when first isolated on culture media.

2 Making a microscopic examination on stained material from an isolated culture of that colony.

3. Performing various tests to obtain a list of the characteristics of the organism.

4. Making a complete identification of the organism.

Subcourse Components:

The subcourse instructional material consists of six lessons as follows:

Lesson 1, Introduction.

Lesson 2, Microscopic Examination and Cultivation of Bacteria.

Lesson 3, Common Tests and Gram-Positive Cocci.

Lesson 4, Gram-Negative Cocci; Gram-Positive Bacilli.

Lesson 5, Enterobacteriaceae.

Lesson 6, Other Pathogenic Gram-Negative Bacilli and Antibiotic Sensitivity Tests.

Here are some suggestions that may be helpful to you in completing this subcourse:

--Read and study each lesson carefully.

--Complete the subcourse lesson by lesson. After completing each lesson, work the exercises at the end of the lesson, marking your answers in this booklet.

--After completing each set of lesson exercises, compare your answers with those on the solution sheet that follows the exercises. If you have answered an exercise incorrectly, check the reference cited after the answer on the solution sheet to determine why your response was not the correct one.

Credit Awarded:

Upon successful completion of the examination for this subcourse, you will be awarded 20 credit hours.

To receive credit hours, you must be officially enrolled and complete an examination furnished by the Nonresident Instruction Branch at Fort Sam Houston, Texas.

You can enroll by going to the web site http://atrrs.army.mil and enrolling under "Self Development" (School Code 555).

LESSON ASSIGNMENT

LESSON 1	Introduction.
TEXT ASSIGNMENT	Paragraphs 1-1 through to 1-22.
LESSON OBJECTIVES	Upon completion of this lesson, you should be able to:

1-1. Match the name of a symbiotic association mutualism, commensalism, or parasitism with its description.

1-2. Associate basic terms of parasitology with their definitions.

1-3. Identify the means by which bacteria reproduce.

1-4. Identify the functions of the bacterial capsule, flagellum, cell wall, and spore.

1-5. Given the description of a toxin; identify its category.

1-6. Classify bacteria according to their source of nutrients.

1-7. Given a common microscopic morphological description; identify the corresponding morphological term.

1-8. Identify the proper methods for collecting, processing, and shipping bacteriological specimens.

SUGGESTION After reading and studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 1

INTRODUCTION

Section I. HOST-PARASITE RELATIONSHIPS

1-1. CAUSATION OF MICROBIAL DISEASE

Microbial disease must be understood in terms of the interrelationship among parasites, the host, and the environment. For example, the general health of the host may determine whether a disease occurs and how severe it .may be. Even though a microorganism may be part of the ordinary flora, it can cause disease if the host's natural defenses are not fully effective. Some common symbiotic relationships between two organisms, such as man and a microorganism, are given below.

a. **Mutualism.** In this case, both organisms benefit.

b. **Commensalism.** Commensalism is a relationship where one organism benefits without producing harmful effects for the other.

c. **Parasitism.** In parasitism, one organism benefits at the expense of the other. The parasite may be facultative or obligatory. If the parasite is able to live independently of the host, it is considered a facultative parasite. When the parasite has an absolute requirement for a host and cannot live outside the host, it is an obligatory parasite. In the microbial association of parasitism, disease results if the host is injured by the parasite. In this regard, the parasite has certain characteristics favoring its establishment in the host. On the other hand, the host has certain characteristics that oppose the establishment of the parasite. The outcome of the host-parasite relationship depends on the active interplay between factors.

1-2. TERMINOLOGY

a. Parasites that usually cause disease are called pathogens, and pathogenicity is the term used to denote disease-producing ability. Virulence denotes the degree of pathogenicity; it is used to describe a particular strain or variety of a species. Pathogenicity or virulence may be due to the bacteria's invasiveness or toxigenicity. Invasiveness means the ability of the bacteria to enter, spread, and multiply in host tissue. Toxigenicity refers to the ability of the bacteria to produce toxic substances.

b. Virulence is usually expressed by the LD_{50} that is, the concentration of microorganisms representing a lethal dose for 50 percent of the experimental animals under standardized conditions. The LD_{50} is specific to the laboratory animal employed.

c. The process whereby a pathogenic microbe enters into a relationship with the host is known as infection. The infection mayor may not result in overt-disease. In this regard, the host may overcome the infection, the host may develop latent, that is, unapparent infection, or the host may become a healthy carrier of the pathogen.

d. Bacteria reproduce asexually by splitting at right angles to their long axis-a process called binary fission.

1-3. THE INFECTIOUS PROCESS

Various steps are required for infectious process. First, an appropriate portal of entry is required. The respiratory tract, the gastrointestinal tract, direct contact, and insect bites may each represent the portal of entry. The portal of entry is usually specific for any particular pathogen. Second, the pathogen must establish itself in and reproduce within the host. This involves its spread through the host's tissues via the lymphatic and the blood stream. Third, to complete the transmission of the pathogen to a new host, a portal of exit is required. The portal of exit may be the respiratory tract, the gastrointestinal tract, direct contact, or an insect bite. Finally, to ensure perpetuation of the parasite, the pathogen must be introduced to a new host. This may occur by the ingestion of contaminated food or drink, by direct contact with infected persons, by contact with contaminated objects (fomites), or by-insect vectors.

Section II. ASPECTS OF PATHOGENICITY

1-4. CAPSULATION

Of primary consideration is the presence of a capsule on most pathogenic bacteria. Encapsulated bacteria are able to resist phagocytosis by leukocytes better than nonencapsulated bacteria. The loss of the capsule in certain bacteria (pneumococci, Friedlander's bacillus) results in the loss of virulence. The capsule is generally regarded as a factor of invasiveness.

1-5. BACTERIAL TOXINS

Bacterial toxins are known to play a role in the ability of the bacteria to cause disease. Toxins are divided into endotoxins and exotoxins.

a. **Exotoxins.** Exotoxins are secreted by bacteria into their environment and are potent and specific in their action upon host tissue. The ability to form exotoxins is usually attributed to certain gram-positive rods such as the clostridia and the corynebacteria. The exotoxin has been found to be a highly antigenic, heat labile, proteinaceous substance that is usually destroyed by proteolytic enzymes. However, the very potent and toxic exotoxin of Clostridium botulinum is the exception; is not destroyed by proteolytic enzymes. The toxic properties, but not the antigenic properties, of exotoxins are destroyed by formalin, heat, or prolonged storage. Such treated toxins are called toxoids and find great practical use in immunization programs.

b. **Endotoxins.** Endotoxins are found intracellularly, and are considered to be part of the bacterial cell wall. They are released upon destruction of the bacterial cell. Endotoxins are usually associated with gram-negative bacteria, and considered to be weakly antigenic. Among their other properties, endotoxins are heat stable polysaccharides that are not digested by proteolytic enzymes. Endotoxins are weakly toxic and induce a generalized reaction in the host that manifests itself in the form of a febrile response. Endotoxins may also cause shock.

1-6. EXTRACELLULAR ENZYMES

Certain microorganisms produce extracellular enzymes that contribute to their pathogenicity.

a. Production of the enzyme coagulase has been correlated to the pathogenicity of the staphylococci. Coagulase acts upon plasma causing it to coagulate. This activity results in the walling off of the site of infection and causes a layer of fibrin to form on the cell wall of the bacteria, which enables the staphylococci to resist phagocytosis.

b. Collagenase is an enzyme that is produced by some of the clostridia. It acts upon collagen, a constituent of connective tissue. The breakdown of collagen promotes the spread of bacteria in tissue.

c. Hyaluronidase is an enzyme that is known as the spreading factor. It acts upon hyaluronic acid, a constituent of connective tissue. The enzyme is produced by staphylococci, clostridia, streptococci, and pneumococci. The activity of hyaluronidase contributes to the spread of the pathogens through tissue.

d. Streptokinase, also known as fibrinolysin, is an enzyme produced by streptococci, staphylococci, and Clostridium perfringens. Streptokinase activates a proteolytic enzyme of plasma known as plasmin. Plasmin is able to dissolve coagulated plasma, and this activity may aid in the spread of the bacteria.

e. Hemolysins are a group of soluble substances produced by staphylococci, pneumococci, some clostridia, and groups A and C of the blood cells and probably streptococci. These hemolysins destroy red tissue cells.

f. Leukocidins are substances that destroy leukocytes; they are produced by streptococci and staphylococci.

Section III. HOST RESISTANCE

1-7. NONSPECIFIC FACTORS

a. Skin and mucous membrane barriers.

(1) Physical barriers.

(a) Intact skin.

(b) Mucous-sticky lining.

(c) Nasal hair.

(d) Cilia.

(e) Peristaltic action.

(f) Normal flora-occupying attachment sites.

(2) Chemical barriers.

(a) Acid pH (stomach skin).

(b) Bile salts (intestine}.

(c) Lysozyme (eyes).

(3) Microbial antagonism.

(a) Bacteriocins from normal flora.

(b) Antimicrobial factors from serum.

(c) Competition for nutrients.

b. Phagocytosis.

(1) PMN, monocytis, and macrophages ingest foreign particles in the host.

(2) Numerous enzymes act to degrade ingested particles.

(3) Local tissue damage-inflammation may occur.

c. Emotional and nutritional states (hormones, vitamins, etc.) play an undefined role in resistance.

1-8. SPECIFIC FACTORS-IMMUNOLOGICAL RESPONSE

a. **Antibodies.**

(1) Neutralize the antigen.

(2) Opsonize the antigen.

b. **Cell mediated immunity.**

(1) Antigen stimulates the release of biologically active substances called lymphokines.

(2) Lymphokines enhance phagocytosis and killing.

c. **Complement system.**

(1) Complex system of serum proteins.

(2) Assist antibody in neutralizing the antigen.

(3) Assist antibody in lysing bacterium.

d. **Interferon.**

(1) Cell protein-local production.

(2) Production stimulated by viruses and other microbes.

(3) Local defense against antigens.

Section IV. THE BACTERIAL CELL

1-9. AUTOTROPHIC BACTERIA

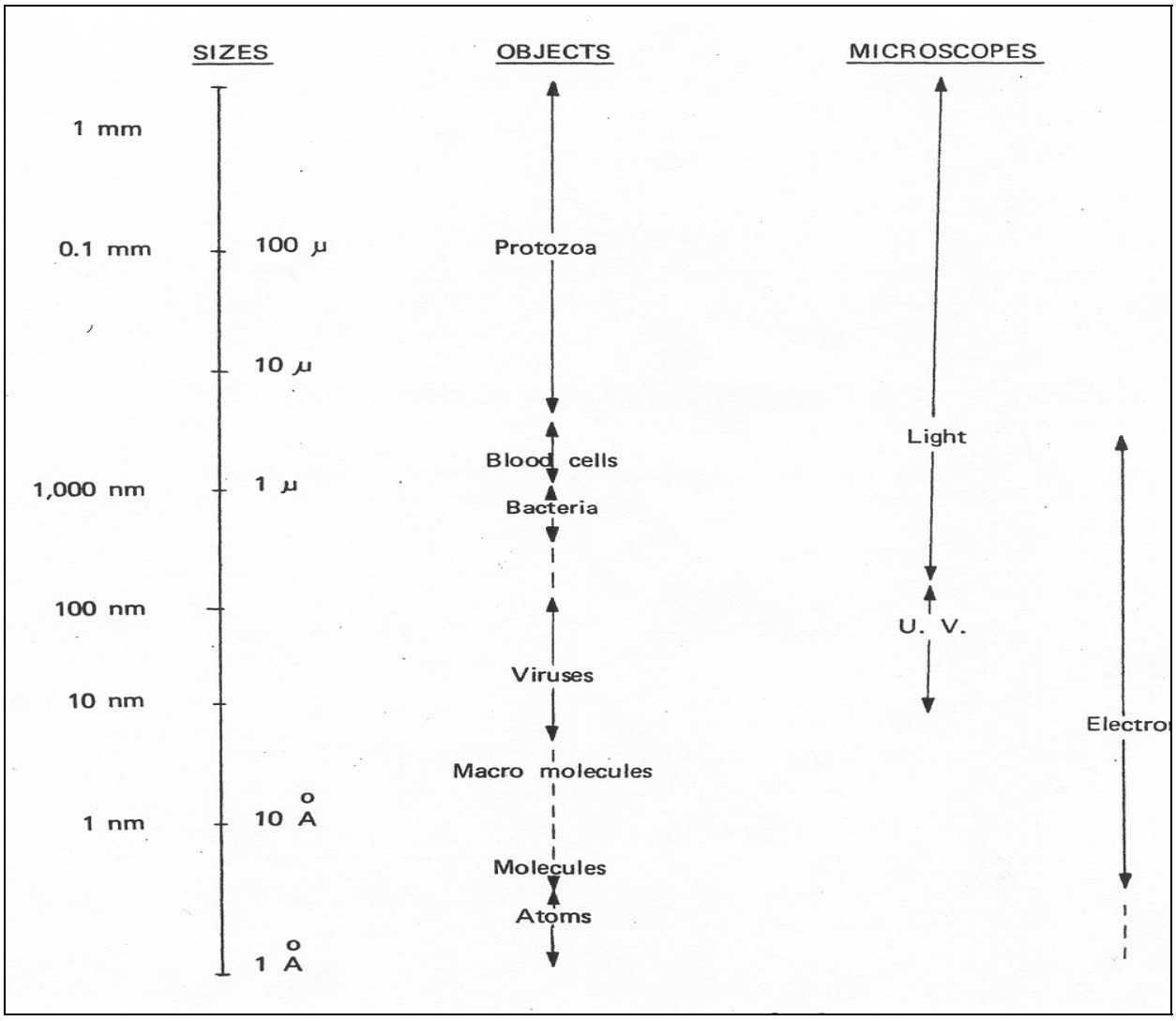
Autotrophic bacteria obtain energy and grow on inorganic media, employing carbon dioxide (CO_2) as their sole source of carbon. Autotrophs begin with CO_2 and ammonia (NH_3) and from these build an entire protoplasmic structure of protein, fat and carbohydrates, using the oxidation of ammonia to obtain energy for their other processes.

1-10. HETEROTROPHIC BACTERIA

Heterotrophic bacteria obtain energy from organic carbon sources. Heterotrophs require the addition of sugars, amino acids, purines, pyrimidines, and vitamins to their culture media. The fermentation of sugar is their primary source of energy. Parasitic bacteria are heterotrophs. They have become adapted to an environment in which many kinds of organic materials are normally available. In many cases such organisms have lost their ability to synthesize certain complex organic substances needed for their growth. Bacterial parasites require nutrients from living organisms and may cause harm to the host. Saprophytic bacteria are heterotrophs that utilize decaying organic matter for nutrients and usually do not harm the host.

1-11. MORPHOLOGY

The size of bacteria (figure 1-1) is measured in thousandths of a millimeter. Generally, the following dimensions apply. The coccus has a diameter of approximately 1 micron (μ); the bacillus appears as a rod with a width of 0.5 μ and a length of 2 μ; and the spirochete appears as a corkscrew with a width of 0.2 μ; and a length of 10 μ. Bacteria can occur in a number of arrangements and a predominant arrangement is usually specific for a bacterium. In this regard, prefixes are added to the word indicating the shape of the bacterium.



NOTE: 1mm = 1,000 µ; 1 µ = 1,000 nm; 1 nm = 10 Å (Å = Angstrom unit)

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Figure 1-1. Size range of objects with different types of microscopes.

a. **Coccus (Plural, Cocci).** These are spherical bacteria occurring in any of the following arrangements:

(1) Singly (coccus) (figure 1-2A).

(2) In pairs (diplococcus) (figure 1-2B).

(3) In chains (streptococcus) (figure 1-2C).

(4) In clusters (staphylococcus) (figure 1-2D).

(5) In clusters of 4 (tetrad) or 8 (cube) (figure 1-2E).

b **Bacillus. (Plural, Bacilli).** These are rod-shaped bacteria occurring in any of the following arrangements.

(1) Singly (bacillus) (figure 1-2F).

(2) In pairs (diplobacillus) (figure 1-2G).

(3) In chains (streptobacillus) (figure 1-2H).

(4) In palisades (palisade) (figure 1-2I).

c. **Spirillum (Plural, Spirilla).** These are spiral, corkscrew-shaped organisms whose long axes remain rigid while in motion. A spirochete is a spiral microorganism whose long axis flexes when it is in motion (figure 1-2J).

d. **Coccobacillus.** This is a short, plump bacillus with rounded ends, resembling a coccus in shape and arrangement.

e. **Vibrio.** This is a comma-shaped bacillus resembling the spirillum because of its motility.

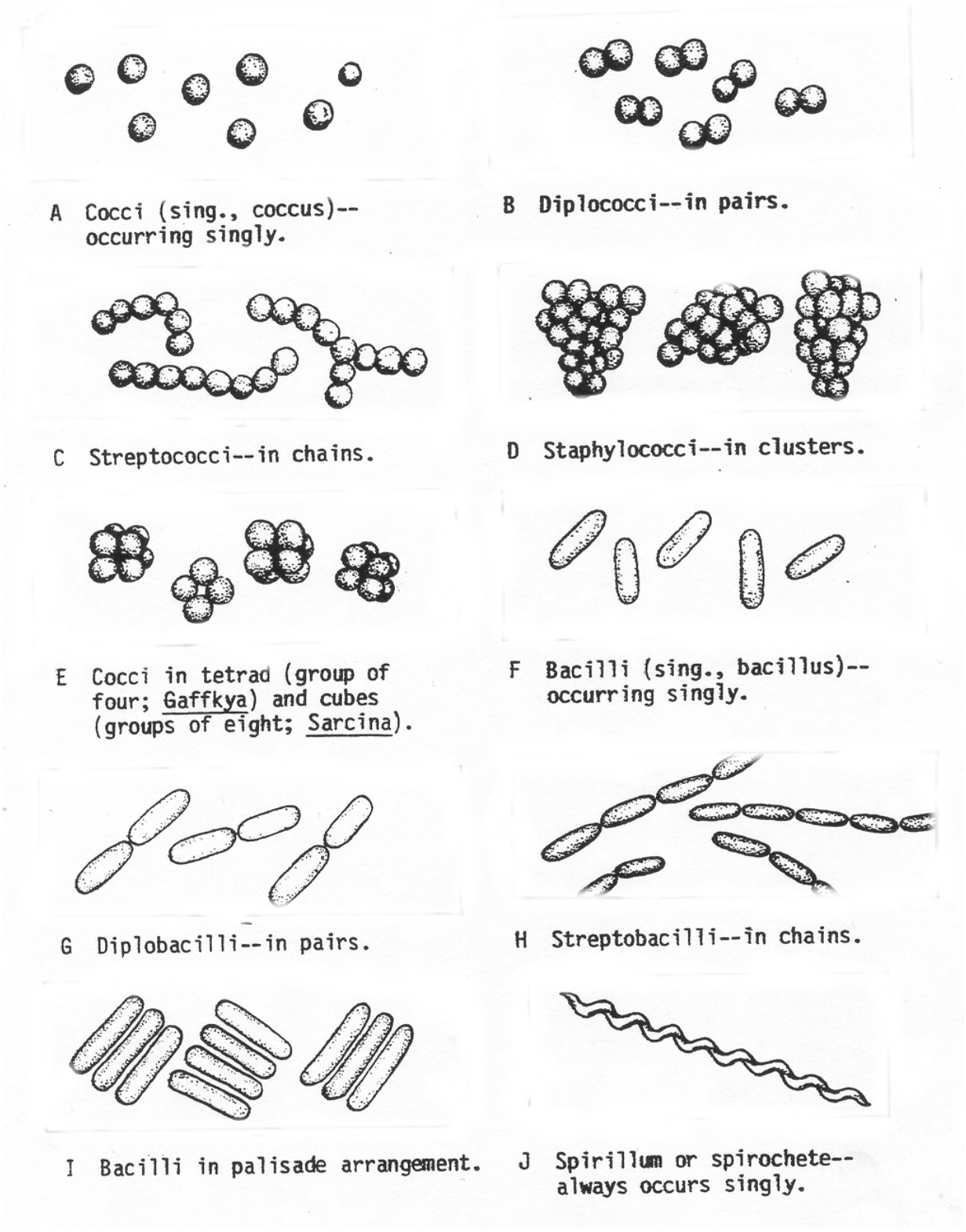

A Cocci (sing., coccus)--occurring singly.
B Diplococci--in pairs.
C Streptococci--in chains.
D Staphylococci--in clusters.
E Cocci in tetrad (group of four; Gaffkya) and cubes (groups of eight; Sarcina).
F Bacilli (sing., bacillus)--occurring singly.
G Diplobacilli--in pairs.
H Streptobacilli--in chains.
I Bacilli in palisade arrangement.
J Spirillum or spirochete--always occurs singly.

Figure 1-2. Shapes and arrangements of bacteria.

1-12. STRUCTURE OF BACTERIA

The typical structure of a bacterial cell is shown in figure 1-3. It consists of the following general structures, found in all bacterial cells; and special structures, found in specific types of bacterial cells:

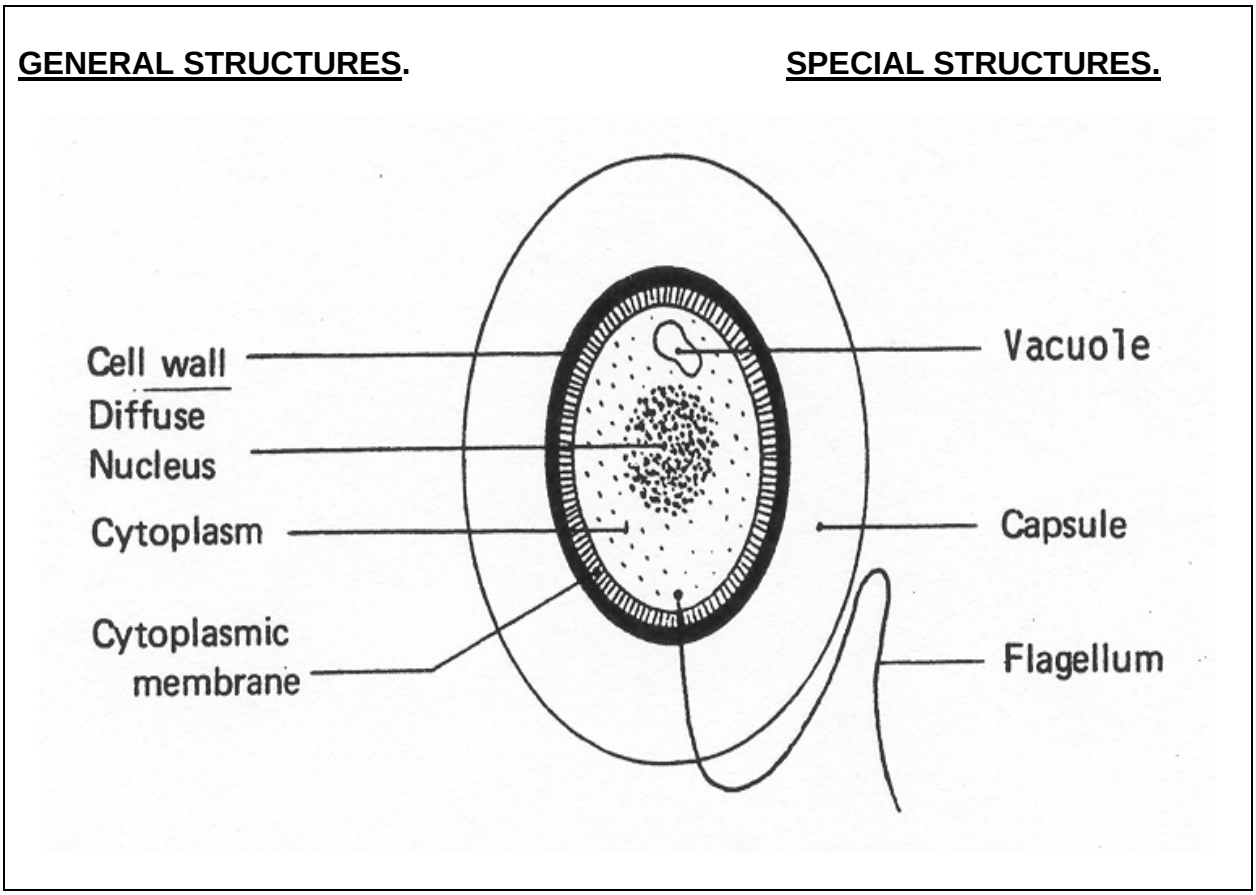


Figure 1-3. Bacterial cell structure.

a. **General Structures.**

(1) Cell wall. A thin, rigid cellulose covering that encloses the protoplasm of the cell and gives rigidity to the bacterial shape.

(2) Cytoplasmic membrane. A semi-permeable membrane that is located directly beneath the cell wall and which governs osmotic activity.

(3) Cytoplasm. The protoplasmic or vital colloidal material of a cell exclusive of the nucleus.

(4) Nucleus. Diffused chromatin material responsible for replication of the cell. The bacterial cell does not have a nuclear membrane or a well-defined nucleus.

b. **Special Structures.**

(1) Capsule. An accumulation of high molecular weight, excretory substances (slime layer) around a bacterium or bacteria. A capsule serves as a defense mechanism against phagocytosis by white blood cells and penetration by viruses.

(2) Flagellum. A protoplasmic strand of elastic protein originating in the cytoplasm and extending from the body of the cell. A flagellum serves as an organ of locomotion. The arrangement of flagella (plural) is peculiar to the species.

(3) Spore. Metabolically resistant body formed by a vegetative bacterium to withstand an unfavorable environment. Only bacilli form spores. The position and size of a spore within a bacillus is peculiar to the species.

(4) Inclusion bodies. Vacuoles of reserve or waste materials contained within the cytoplasm.

1-13. IDENTIFICATION OF BACTERIA

Since there are several thousand species of bacteria, it would be impossible to identify them on the basis of appearance alone. Therefore, the bacteriologist employs a wide variety of techniques, based upon known characteristics of specific bacteria, to arrive at the identity of a given specimen. The following characteristics, which are used frequently as terms of reference, assist the microbiologist in the positive identification of bacteria as well as in eliminating them from consideration.

a. **Food Requirements.**

(1) Natural media.

(a) Saprophytes - grow on dead organic matter.

(b) Parasites - grow on living tissue.

(2) Artificial media.

(a) Grow on any culture medium.

(b) Grow only on special culture media.

(c) Will not grow on any artificial culture medium.

b. **Oxygen Requirements.**

(1) Aerobes -- grow in the presence of free oxygen.

(2) Anaerobes -- grow without free oxygen

(3) Obligate - aerobes -- must have free oxygen for growth.

(4) Obligate aerobes -- must not have free oxygen.

(5) Facultative aerobes -- able to adjust to an aerobic environment.

(6) Facultative anaerobes -- able to adjust to an anaerobic environment.

(7) Microaerophiles -- require small amounts of free oxygen for growth.

c. **Colony Morphology.**

d. **Microscopic Examination.**

(1) Size.

(2) Shape.

(3) Spore formation - sporeformers or nonsporeformers.

(4) Capsule formation - encapsulated or nonencapsulated.

(5) Motility - motile or nonmotile.

(6) Staining characteristics. Specimens are normally stained prior to microscopic examination. Various species react differently to the stains.

(a) Gram-positive - bacteria which, when stained by the gram stain method, retain the crystal violet stain (purple or blue).

(b) Gram-negative - bacteria which, when stained by the gram stain method, do not retain the crystal violet stain, but retain the color of the counterstain (red).

(c) Acid-fast - bacteria which, when stained with certain dyes and then treated with an acid, followed by a counterstain, retain the color of the dye.

(d) Nonacid-fast - bacteria which, when treated as in (c), above, retain the counterstain rather than the dye.

e. **Pathogenicity.**

(1) Hemolytic or nonhemolytic.

(a) Beta hemolytic - can cause complete hemolysis (dis- (solution) of red blood cells.

(b) Alpha hemolytic - cause partial hemolysis of red blood cells.

(c) Gamma forms - do not cause hemolysis.

(2) Production of toxins.

(a) Exotoxins - extremely potent poisons which are produced in bacterial cells and which diffuse freely into the cells of host tissues, causing severe systemic poisoning.

(b) Endotoxins - toxins, less potent than exotoxins, which are produced in bacterial cells and which diffuse into the host cells only after the bacterial cell disintegrates.

1-14. PATHOGENIC BACTERIA

Table 1-1 presents a list of the principal pathogenic bacteria of public health importance, organized in such a way as to illustrate the aids in identification discussed in the above paragraph.

IDENTIFICATION GROUP	SPECIES	CAUSATIVE AGENT OF	REMARKS
GRAM-POSITIVE COCCI			
Alpna hemolytic	Diplococcus pneumoniae	Lobar pneumonia	Produces exotoxin causing skin rash.
Beta hemolytic	Streptococcus pyogenes	Impetigo, septic sore throat, scarlet fever	
	Staphylococcus aureus	Upper respiratory infections, boils, surgical infections, food poisoning	Produces exotoxin causing food poisoning.
GRAM-NEGATIVE COCCI	Neisseria gonorrhoeae	Gonorrheal conjunctivitis	Kidney-shaped diplococci.
	Neiseseria meningitides	Epidemic cerebrospinal meningitis	Kidney-shaped diplococci.

Table 1-1. Pathogenic bacterial of public health importance (continued).

IDENTIFICATION GROUP	SPECIES	CAUSATIVE AGENT OF	REMARKS
GRAM-POSITIVE BACILLI	Corynebacterium diphtheriae	Diptheria	Produces powerful exotoxin causing inflammation of mucosa and impairment of vital organs.
Aerobic	Bacillus anthracis	Anthrax (Chiefly in herbivorous animals, but also in man)	Forms capsule.
Aerobic,spore-forming	Mycobacterioum tuberculosis	Tuberculosis (man)	
	M. bovis	Tuberculosis (cattle and man)	
	M. leprae	Leprosy	
Aerobic, acid-fast	Clostridium botulinum	Food poisoning	Produces powerful, lethal exotoxin.
	C. tetani	Tetanus (lockjaw)	
Aerobic,spore-forming	C. perfringens	Gas gangrene food poisoning	Produces powerful, lethal exotoxin.

Table 1-1. Pathogenic bacterial of public health importance (continued).

IDENTIFICATION GROUP	SPECIES	CAUSATIVE AGENT OF	REMARKS
GRAM-NEGATIVE BACILLI			
Saprohytic enterics	Eschrichia coli Enterobacter aerogenes Proteus vulgaris Pseudomonas aeruginosa Alkaligenes faecalis Klebsiella pneumoniae	Part of the normal flora of the adult intestinal tract, but pathogenic to infants or when introduced into other parts of the body.	All nonspore-forming.
Pathogenic entrics	Salmonella typhi S. paratyphi S. typhimurium	Typhoid fever Partyphoid fever Acute gastroenteritis (Salmonellosis- "food poisoning"	
	Shigella dysenteriae[1] S. flexnerei	Bacillary dysentery	Produces a somatic endotoxin. [1]Also produces a paralytic exotoxin.
	S. sonnei[2]	[2]Infant diarrhea	Motile, comma-shaped.
	Vibrio cholerae	Cholera	

Table 1-1. Pathogenic bacterial of public health importance (continued).

IDENTIFICATION GROUP	SPECIES	CAUSATIVE AGENT OF	REMARKS
SMALL GRAM-NEGATIVE BACILLI			
Non-motile, nonspore-forming	Brucella abortus[1]	Contagious abortion in animals	[1]Occurs in cattle.
	B. suis[2]		[2]Occurs in swine.
	B. melitensis[3]		[3]Occurs in sheep
	Hemophilus influenzae	brucellosis (undulant fever) in man	Encapsulated.
	H. pertussis (Bordetella)	Pharyngitis, otitis, sinusitis, pneumonitis	
	H. ducreyi	Whooping cough	
	Pasteurella (yersinia)	Chancroid	
	pestis	Plague	
	F. tularensis	Tularemia	
SPIROCHETES	Treponema pallidum	Syphilis	Does not stain with ordinary stains nor grow on artificial media.
	Borrelia recurrentis	Relapsing fever	
	Leptospirosa ichterohemorrhagiae (also L. canicola, L. autumnalis, and L. pomona)	Leptospirosis (Weil's disease, infectious jaundice)	May be stained and cultured (chick embryo).

Table 1-1. Pathogenic bacterial of public health importance (concluded).

Section V. COLLECTING AND PROCESSING OF BACTERIOLOGICAL SPECIMENS

1-15. BASIC PRINCIPLES

The improper collecting and processing of bacteriological specimens is often responsible for the failure to isolate and identify the bacterial agent of disease. Certain basic principles, therefore, should be followed at all times.

a. First, instruments, containers, and other equipment in direct contact with specimens must be sterile. The cartons for the collection of stool specimens are an exception to this rule.

b. Second, material for culture must not come in contact with chemicals, disinfectants, or germicidal agents.

c. Third, material for culture should be obtained before the patient receives antibiotic therapy. If this is not possible, the type of therapy should be indicated on the bacteriology request form. Such substances as penicillinase should then be added to the media if penicillin is indicated; para-aminobenzoic acid should be added if sulfonamides are indicated.

d. Fourth, specimens should be properly labeled and dated, and should be delivered to the bacteriological section of the clinical laboratory immediately after collection. Specimens should be inoculated to media immediately after delivery to the bacteriology section.

e. Fifth," if anaerobic cultures are requested, the specimen should be inoculated to fluid thioglycollate medium at the time the specimen is collected, or as scion thereafter as possible. Exposure to atmospheric oxygen should be avoided completely if possible. (See discussion of anaerobic methods in Lesson 2, Section IV).

f. Sixth, to avoid contamination, always culture the specimen before making smears or performing special tests.

g. Staining and testing procedures mentioned in the following pages on processing will be explained more completely in the following chapters.

1-16. COLLECTING AND PROCESSING OF BLOOD CULTURES.

NOTE: Refer to figure 1-4.

a. **Circulatory Infections.** Isolation of microorganisms from blood is of value to the medical officer in the diagnosis and treatment of various infections. Blood in man is normally sterile, and the presence of bacteria in the bloodstream is a pathogenic condition. This condition is called septicemia or bacteremia.

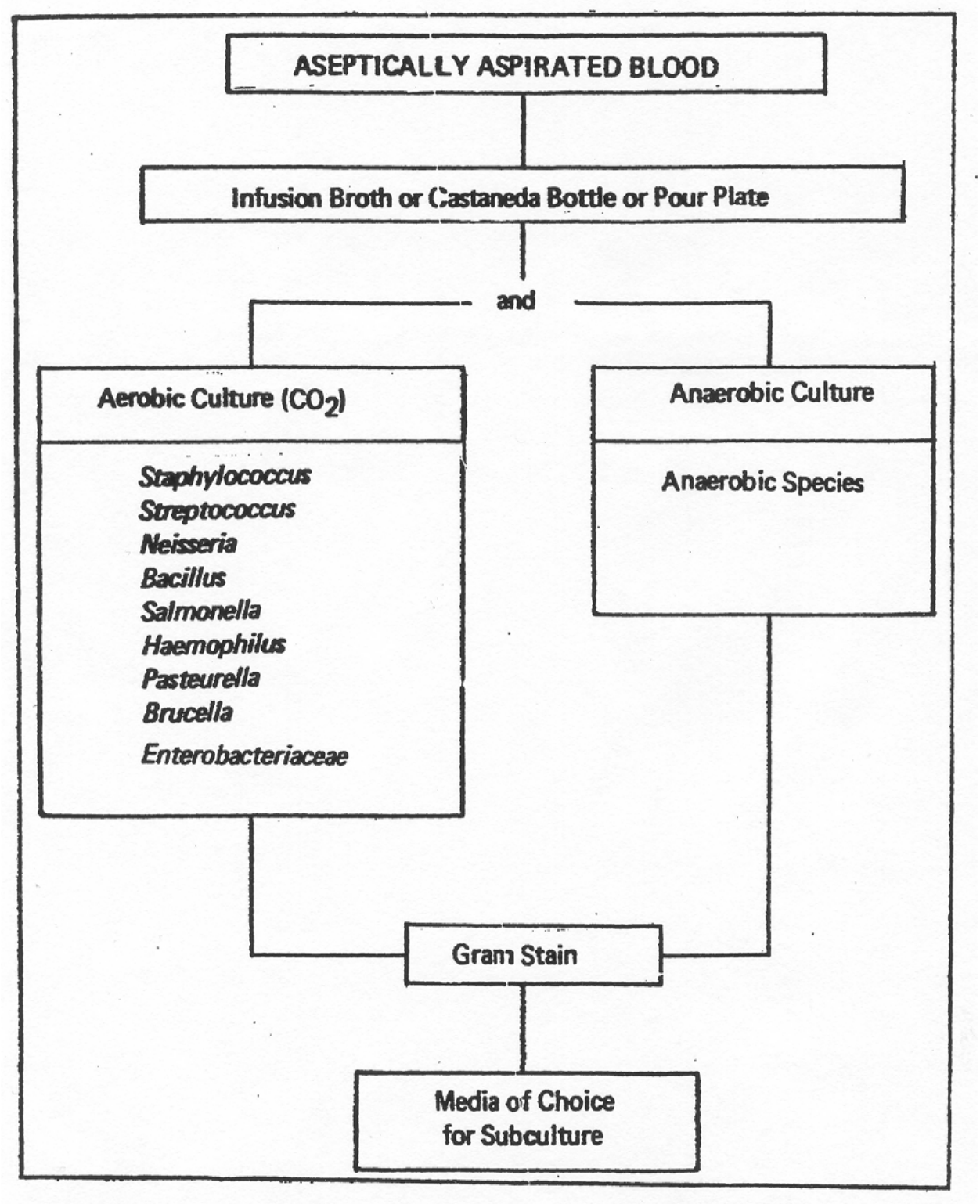


Figure 1-4. Technique for processing blood specimens.

b. **Timing of Collections.** Many blood cultures yield negative results because samples are not collected during the proper stage of the disease concerned. Specimens of blood should be drawn when symptoms are indicative of circulatory involvement, (chills, fever, convulsions, etc.). Negative results from a single blood culture never rule out septicemia.

c. **Preparation of Venipuncture Site.** Preparation of the site for venipuncture is crucial. Swab the site with cotton-tipped applicator sticks saturated with iodine tincture, USP. Allow to dry. Remove with sponges saturated with 70 percent isopropyl alcohol. Then repeat the entire process.

d. **Processing of Specimen.** There are many methods for the culturing of blood specimens. Regardless of the methods of choice, the blood should be aspirated by venipuncture using the strictest aseptic technique. Blood may be immediately inoculated into broth at the bedside of the patient, or the blood may be drawn and transported to the laboratory in sterile, capped test tubes containing anticoagulant.

e. **Prepared Culture Bottles.** Prepared culture bottles are available commercially, which make it possible to withdraw blood and introduce it into the culture medium within a sterile, closed system. One needle is inserted into the vein. The blood passes through a vinyl tube to a needle at the other end of the tube. This other needle has been used to puncture the rubber cap of a vacuum bottle containing the medium. When sufficient blood has passed into the bottle (about 1 ml of blood for each 10 ml of medium), the vinyl tube is pinched to stop the blood flow, the tourniquet is removed, and the needle is removed from the vein. This system can be used both aerobically and anaerobically. For aerobic culture, filtered air is allowed to enter the bottle after blood collection. Frequently included in the medium is the desirable anticoagulant called sodium polyanethol sulfonate (Liquoid).

f. **Culture of Specimen-Infusion Broth Technique.**

(1) Inoculate a volume of approximately 5 ml of blood to each of two bottles or flasks containing the infusion broth. Each bottle or flask should contain 50 ml of trypticase soy broth or other suitable infusion broth.

(2) Incubate one broth aerobically, under CO_2 tension at 37º C

(3) Incubate the second broth anaerobically at 37º C.

NOTE: Trypticase soy broth in vacuum bottle with CO_2 incorporated is available commercially. If inoculated and incubated according to the manufacturer's instructions, this medium will yield excellent results.

g. **Culture of Specimen-Castaneda Bottle Technique.**

(1) Inoculate a volume of approximately 5 ml of blood to each of two Castaneda bottles (these are bottles containing both a broth and an agar surface).

(2) Incubate one bottle aerobically, under CO_2 tension at 37º C.

(3) Incubate the second bottle anaerobically at 37º C.

NOTE: This method is desirable when colonial characteristics are to be studied. Bottles prepared with thioglycollate broth will support growth of anaerobes.

h. **Culture of Specimen--Pour Plate Technique.**

(1) Prepare trypticase soy agar pour plates using the method in Lesson 2, Section II.

(2) Inoculate the desired volume of the patient's blood to the liquid state in the pour plate agar.

(3) Incubate the plate under CO_2 at 37º C. Aerobic and anaerobic conditions will be provided in the pour plate.

NOTE: This method is desirable when hemolysis is to be is to be studies.

i. **Care of Sample.** The blood culture media should be placed in the incubator as soon as possible. Both CO_2 tension and anaerobic conditions (see chapter 4) should be incorporated into the incubation to promote growth and reproduction of the organisms most frequently encountered in septicemia. Blood culture specimens should be incubated at 37º C, and should remain in the incubator and be observed for at least 21 days before being reported as negative and discarded. Blood cultures should be observed daily for signs of growth. At the first sign of turbidity, gas formation, pigmentation of media, "cotton-ball" formation, coagulum formation, or growth on the agar slant, the culture should be checked for the presence of bacteria.

j. **Checking the Culture.**

(1) To check a blood culture for the presence of bacteria, first mix the culture well by gentle swirling. Using a sterile syringe and needle and aseptic technique, remove an aliquot of 1 to 2 ml from the blood culture. Use a portion of the sample to inoculate the appropriate media for subculture, usually blood agar, chocolate agar, and thioglycollate broth. Place another portion of the sample on a clean glass slide and allow to air dry. Perform a gram stain on this slide. (Gram stain procedures are explained in Lesson 3.) If there is any visible evidence of growth in the blood culture, and if organisms are demonstrated on the gram stain cell, call the requesting physician immediately.

(2) If visible signs of growth do not occur in a blood culture, the specimen should still be checked for growth at least twice a week during the three-week incubation period. The procedure to check these cultures is the same as the method used to check cultures with visible signs of growth.

(3) A negative blood culture usually remains quite clear; however, it may develop cloudiness after prolonged incubation. This is usually due to shaking the bottle during each observation with the development of turbidity from fibrin or agar particles. Many times this type of turbidity occurs immediately above the red cell layer and it may suggest bacterial growth. In any case, turbidity alone is not a reliable guide for cellular growth, and a gram stain should always be made to determine the presence of microorganisms.

(4) Failure to examine blood cultures often enough or to hold the cultures long enough may result in delay or failure in recognizing the presence of microorganisms. On the other hand, excessively frequent opening of the culture bottles may result in contamination of the culture. It is necessary that a strictly aseptic technique always be followed in the handling and processing of any culture, particularly a blood culture.

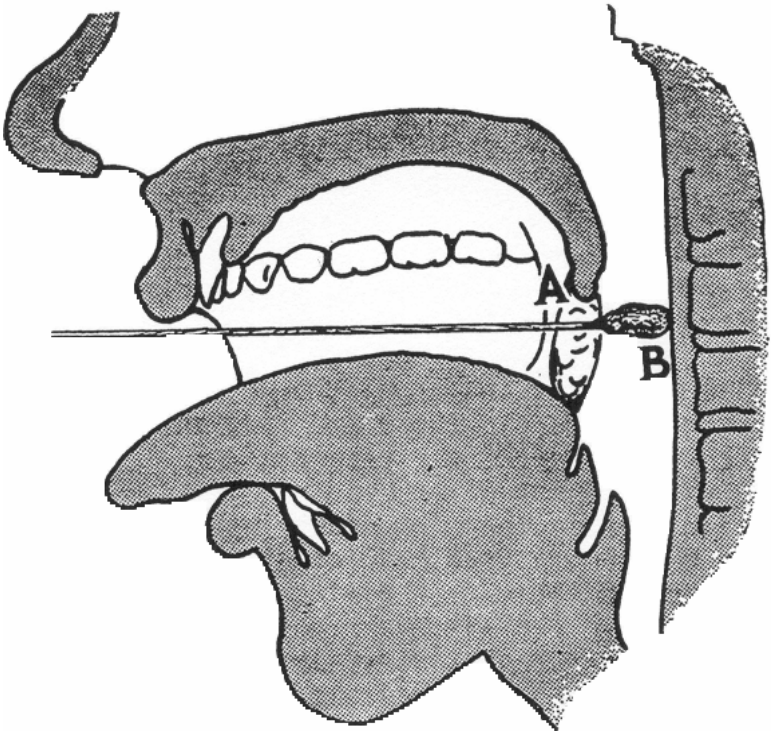
1-17. COLLECTING AND PROCESSING OF SPECIMENS FROM THE RESPIRATORY TRACT

a. **Importance of Throat and Nasopharyngeal Specimens.** Throat and nasopharyngeal cultures are important in the diagnosis of such infections as streptococcal sore throat, scarlet fever, diphtheria, and whooping cough. They are also useful in determining the focal point of infection in such diseases as rheumatic fever and acute glomerulonephritis. In epidemiological studies, these cultures have been essential for the detection of carriers of beta hemolytic streptococcus, staphylococcal infections, Corynebacterium diphtheriae, and other potential pathogens.

b. **Collecting and Primary Care.**

(1) Collect specimens under good lighting using a sterile cotton-tipped applicator stick (see figure 1-5). Depress the tongue with a tongue blade and pass the swab gently over the crypts and tonsils. Also move the swab across the back surface of the pharynx depending on which areas appear to be red and swollen or suggestive of infection by displaying white patchy areas or lesions. Be careful to avoid touching the swab to the tongue, cheek, or teeth as this will result in a culture highly contaminated with a large amount of normal throat flora, and thereby reduce the chances of recovering a true pathogen if one is present.

(2) Place the swab in a sterile test tube and immediately transport the specimen to the clinical laboratory. The sterile tube may contain sterile culture broth to prevent drying of the specimen. Thioglycollate, Trypticase *soy,* or Todd-Hewitt broth is satisfactory for this purpose.



(A) Crypt and tonsil area.

(B) Black surface of pharynx.

Figure 1-5. Technique for performing throat or nasopharyngeal swab.

c. **Processing.** See figure 1-6 for the basic technique for processing throat and nasopharyngeal specimens.

(1) Group A beta hemolytic streptococci are better obtained from throat swabs than from any other infected area. For this reason the blood agar streak plate should be carefully examined after 18-24 hours of incubation at 37º C for the presence of colonies of beta hemolytic streptococci. Any colony that appears to exhibit beta hemolysis should be subcultured by stabbing with an inoculating needle and streaked on a sheep blood agar plate. If a pure culture is not obtained, the process can be repeated. Further identification of these colonies and/or sensitivity studies may then be carried out as indicated.

(2) Normal throat cultures show a predominance of alpha hemolytic streptococci and neisseria. If a culture shows a predominance of organisms that normally occur only in small numbers, this could be of significance and should be reported.

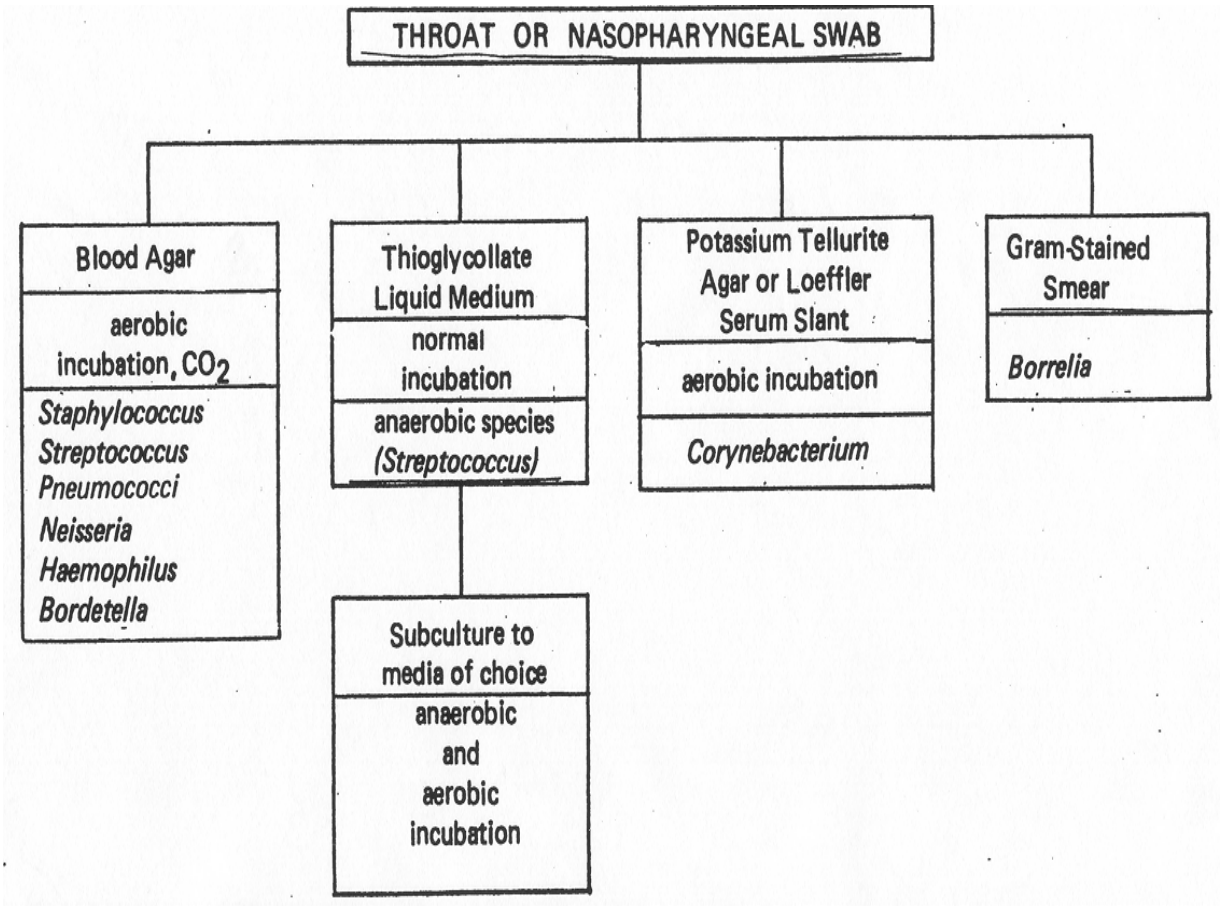


Figure 1-6. Technique for processing throat and nasopharyngeal specimens.

(3) Pneumococci do occur occasionally in the throats of normal individuals; however,' if a large number of the organisms are present, especially in specimens taken from the pediatric age group, it may be of etiological significance and should be reported. A diagnosis of pneumococcal pneumonia may be dependent upon the isolation of pneumococci from a throat or nasopharyngeal culture.

(4) Bordetella pertussis (whooping cough) may also be isolated from the respiratory tract. Cough plates containing a special medium are often utilized; however, nasopharyngeal cultures usually give a higher proportion of positive cultures.

(5) A diagnosis of diphtheria may also be made from throat cultures: however, physicians often expect a definitive diagnosis within 10 minutes based on a direct smear of the throat lesion. Nonpathogenic diphtheria-like (diphtheroid) bacilli may be indistinguishable from the very pathogenic C. diphtheriae. One cannot distinguish these species from a direct smear. The immediate diagnosis of diphtheria is a clinical problem and should rest with the attending physician, with a positive or negative confirmation coming from the bacteriology section after the culture has been processed. Inoculate the specimen on blood agar medium for the growth of aerobes. Inoculate the specimen into thioglycollate medium for the growth of anaerobes and aerobes. Inoculate the specimen on potassium tellurite agar and/or Loeffler's serum slant if Corynebacterium diphtheriae is suspected. Direct smears may be of value if Vincent's angina is suspected. Incubate the blood agar medium under O_2 tension for 18-24 hours at 37º C. Incubate the other media under normal incubation atmosphere for 18-24 hours at 37º C. On potassium tellurite agar, Corynebacterium diphtheriae usually requires 48 hours of incubation at 37º C. If growth is observed in thioglycollate liquid medium, subculture this growth under anaerobic and aerobic conditions.

d. **Sputum Specimens.** Sputum for bacteriological examination (figure 1-7) should be collected in sterile, wide-mouth, screw-capped jars or with the Falcon sputum collection kit. In the case of pneumonia, one sputum specimen is usually sufficient for the examination. This specimen should be collected as soon as the patient awakes in the morning. In the case of pulmonary tuberculosis, a 24-hour collection of sputum or a 3-day collection should be submitted for the examination. Sputum should be processed as soon as possible after it is collected.

(1) Technique for routine organisms and pneumococci.

(a) Inoculate the specimen on blood agar medium for the growth of aerobes. If pneumococci are suspected, place an Optochin disc (ethylhydrocupreine hydrochloride) on the initial streak.

(b) Inoculate the specimen into thioglycollate liquid medium for the growth of aerobes and anaerobes.

(c) Direct smears should be gram-stained and examined for the presence of Staphylococcus, Pneumococci, yeast cells, etc.

(d) Incubate the media for 18-24 hours at 37º C. CO_2 tension may be incorporated to facilitate aerobic growth on the blood agar medium. If pneumococci are present, a zone of inhibited growth will be seen around the Optochin disc after incubation.

(e) If liquid anaerobic growth is observed in the thioglycollate liquid medium, subculture this growth under anaerobic conditions.

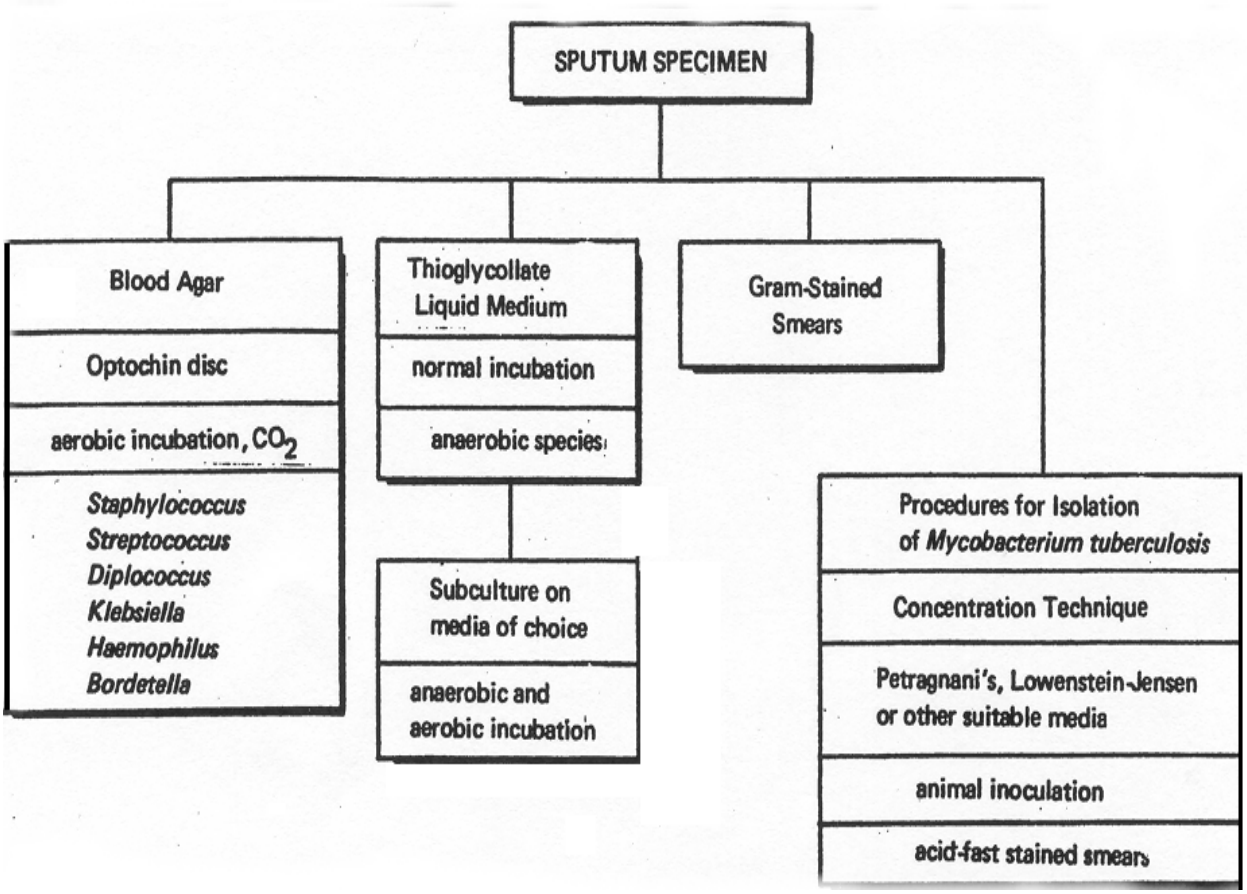


Figure 1-7. Technique for processing sputum specimens.

(2) Technique for acid-fast bacilli.

(a) Prepare smears from grayish or yellowish cheesy masses or purulent blood-tinged portions of the specimen.

(b) Acid-fast stain smears should be prepared and examined. (These are explained in para 2-2)

(c) If the specimen is to be cultured, inoculate a portion of the specimen or Lowenstein-Jensen, Petragnani's, or other suitable media for the cultivation of acid-fast bacilli. A concentrated sediment is generally more reliable than using direct smears. If there is only a small amount, the entire specimen could be used for inoculating the medium. Also, animal virulence tests may be required.

1-18. COLLECTING AND PROCESSING URINE SPECIMENS

a. **Importance.** Urine cultures (figure 1-8) are of value in diagnosing primary infections of the anterior urinary tract (urethritis), bladder (cystitis), and kidneys (nephritis). Urine cultures are also important in diagnosing certain systemic infections, for the etiological agents are often excreted via the urinary tract. Staphylococcus species, a Streptococcus species, and Neisseria gonorrhea are among the primary etiological agents *of* urethritis while Escherichia, Proteus, and occasionally Pseudomonas species are among the chief causative agents of cystitis. Any one or more of these organisms may be the cause of a bacterial nephritis. Yet, many of the above mentioned organisms may be a common urine contaminant. A medical officer usually does not make a diagnosis on the basis of one urine culture. Rather, repeated isolation of large numbers of a particular organism from a series of urine specimens is evidence for pathogenicity of the organism concerned.

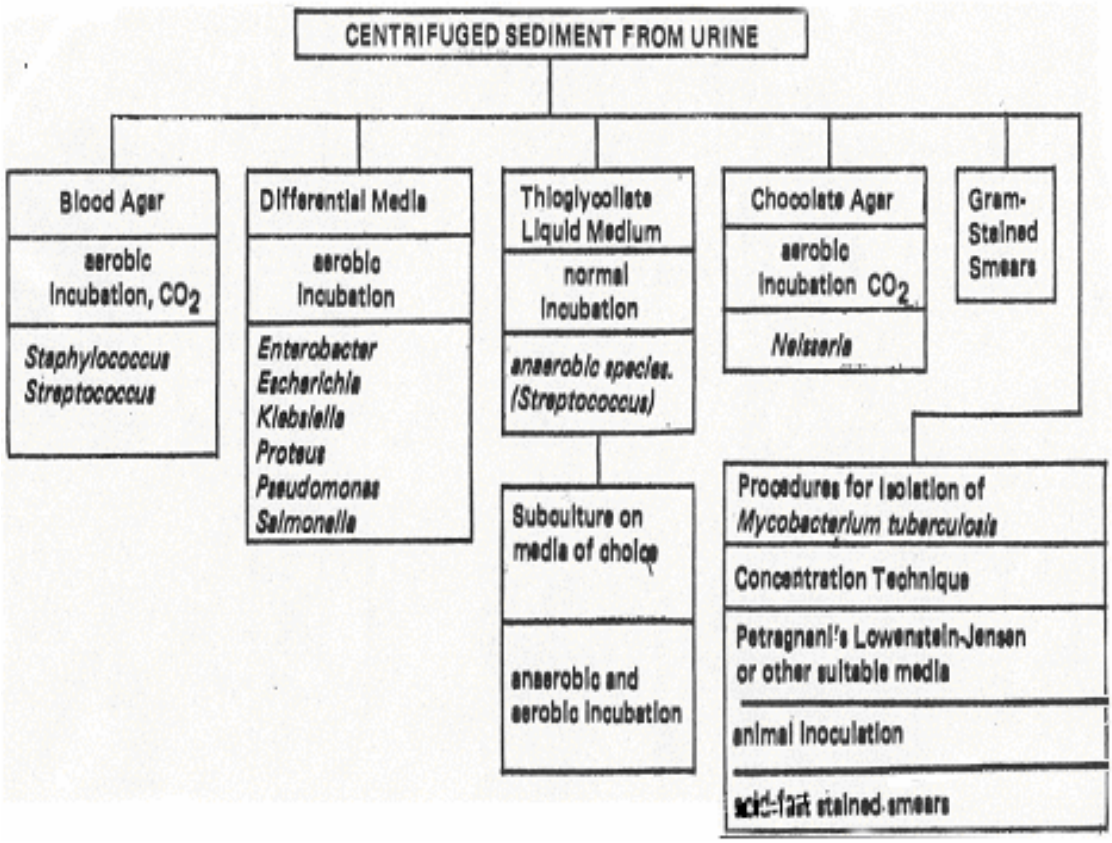


Figure 1-8. Technique for processing urine.

b. **Collection.** "The clean-catch" method of collecting urine is generally preferable. Since the periurethral area (tip of the penis, vulva, and labial folds) is generally contaminated, it must be carefully cleaned prior to collection. Wash carefully with plain soap and water, and rinse. Repent this process, and then rinse well with warm sterile water. The first portion of urine is voided and discarded; this flushes the urethra. A subsequent portion is collected aseptically for culture. All urine specimens for culture should be collected in sterile urine bottles or sterile wide-mouth, screw-capped jars. Delay between the collecting and culturing of urine specimens may contribute to a change in pH and other characteristics of the specimen, resulting in a high death rate of the organisms in question. Urine must be processed within an hour of collection or stored in a refrigerator at 4º C until it can be cultured.

c. **Culture for Routine Organisms.**

(1) Centrifuge the urine specimen and inoculate the concentrated sediment on blood agar, eosin-methylene blue (EMB) or MacConkey's agar, and thioglycollate liquid medium.

(2) If Neisseria is suspected, inoculate a portion of the sediment to supplemented chocolate agar.

(3) Gram-stained smears of the sediment may be helpful.

(4) Incubate the media for 18-24 hours at 37º C. Increased CO_2 tension should be incorporated to facilitate growth on the blood and chocolate agar media.

(5) If growth is observed in the thioglycollate liquid medium subculture this growth under anaerobic and aerobic conditions.

d. **Culture for Acid-Fast Bacilli.**

(1) A 24-hour pooled specimen maybe necessary to demonstrate the presence of acid-fast bacilli in urine. To process this 24-hour collection, add 2-3 grams of tannic acid to the total 24-hour pooled volume and shake well. Place the collection in the refrigerator overnight or for an equivalent period of time. A brown precipitate will form. Decant the supernatant and concentrate the sediment.

(2) The concentrated sediment may be inoculated to suitable media, injected into test animals, and/or spread on clean slides and dried for acid-fast staining. Lowenstein-Jensen or Petragnani's medium is satisfactory for culturing acid-fast organisms.

1-19. COLLECTING AND PROCESSING OF FECAL SPECIMENS

a. **Importance.** The bacteriological examination of fecal specimens (figure 1- 9) aids in the diagnosis of gastrointestinal infections manifested by diarrhea and/or dysentery. Stool cultures, along with blood and urine cultures, are important aids for diagnosing typhoid and paratyphoid fevers. Since many diseases are spread by human carriers through food and drink, properly performed stool cultures on all food-handling personnel strongly supplement public health control measures.

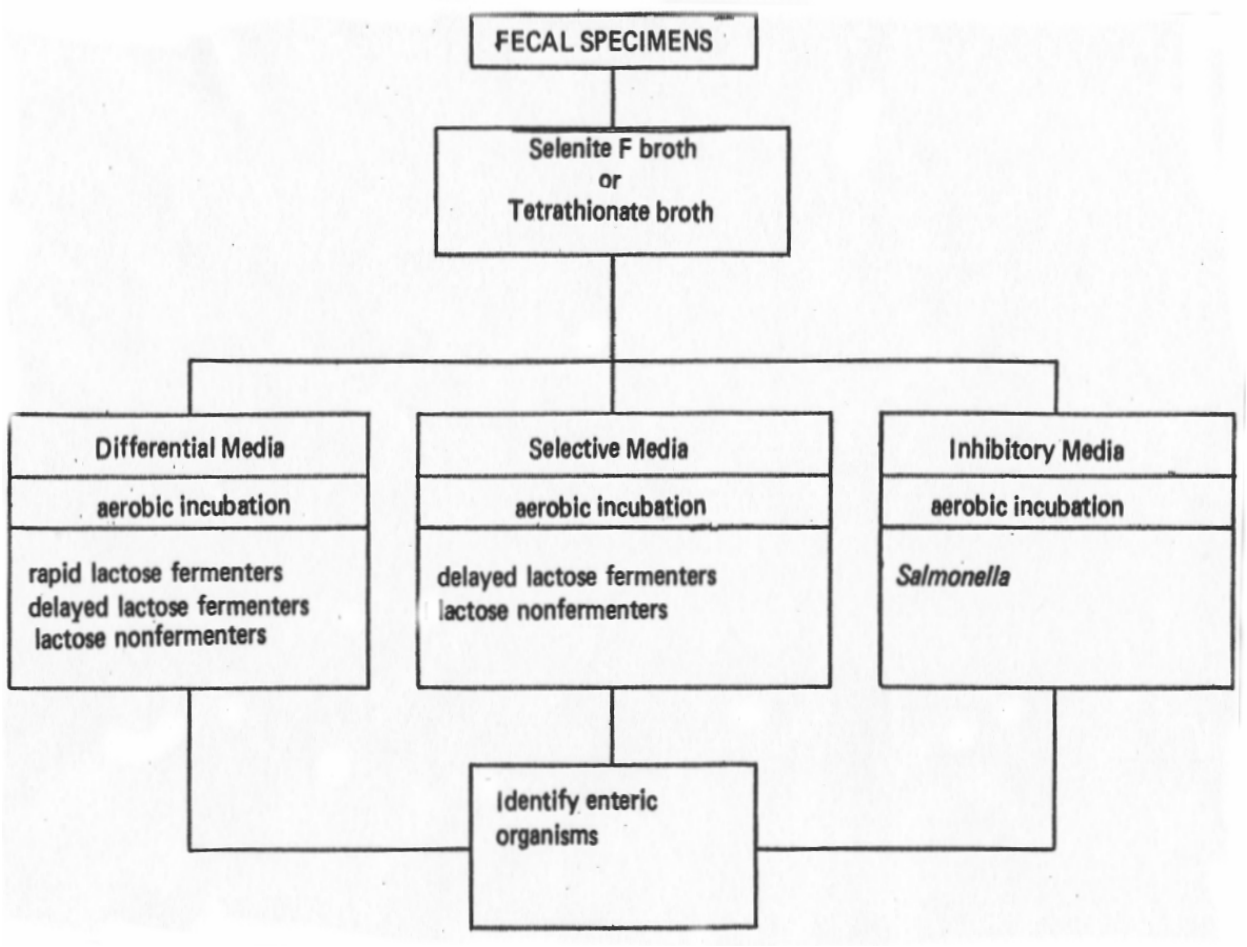


Figure 1-9. Technique for processing fecal (stool) specimens.

(1) The organisms most frequently involved in enteric infections are the Salmonella and Shigella species of the Enterobacteriaceae family. The normal intestinal flora of the adult is composed primarily of anaerobic, gram-negative rods. The biochemical activities of this normal flora can harm species of the family Enterobacteriaceae, which are the focus of clinical testing. Pseudomonas aeruginosa, Alcaligenes faecalis, and Proteus species are sporadically present in the intestine and may be called transient saprophytes. Saprophytic organism (including E. coli, Morganella morganii, and Providencia) have been implicated as etiological agents of infant diarrhea.

(2) In culturing specimens of intestinal origin, the basic problem is the isolation of pathogenic agents from specimens heavily contaminated with saprophytic organisms. To accomplish this, special differential, selective, and inhibitory media are available.

b. **Collection.** Fecal specimens should be collected in clean, wide-mouth containers with tight fitting lids: screw-capped jars are satisfactory. These containers do not have to be sterile but should always be very clean. If rectal swabs are obtained, these should be transported to the clinical laboratory in clean, cotton-plugged test tubes. Rectal swabs may be useful when dealing with infants or large numbers of patients. Fecal material should be cultured as soon as possible after, collection; if culturing is delayed, the isolation of causative agents, particularly Shigella species will be jeopardized.

c. **Culture for Routine Organisms.**

(1) Inoculate sufficient fecal material into selenite F broth (or tetrathionate broth) to produce a heavy suspension. Incubate the broth for 8-12 hours at 37º C. This procedure will boost the growth and reproduction of enteric pathogens while inhibiting the growth and reproduction of enteric saprophytes for approximately 8-12 hours.

(2) After 8-12 hours of incubation, subculture from the selenite F into the following media:

(a) Differential medium, e.g., EMB, MacConkey's, or deoxycholate media.

(b) Selective medium, e.g., Salmonella-Shigella (SS) or deoxycholate-citrate media.

(c) Inhibitory brilliant green medium, e.g., bismuth sulfite or brilliant green media.

(3) Incubate the differential, selective, and inhibitory media aerobically at 37º C.

(4) After 12-16 hours of incubation, examine the differential, selective and brilliant green media for colonies of lactose-nonfermenting organisms (encompassing possible pathogens). These media should be incubated for 24 hours before being discarded as negative.

(5) After 12-24 hours of incubation, examine the bismuth sulfite medium for typical colonial growth of pathogens, particularly Salmonella typhi. The bismuth sulfite medium should be incubated for 48 hours before being discarded as negative.

(6) Isolation of lactose-fermenting organisms from fecal material is; usually considered insignificant, with the exception of isolates from infant diarrhea.

d. **Culture for Streptococcus faecalis.**

(1) Streptococcus faecalis. (SF) medium should be immediately inoculated if the enterococcus Streptococcus faecalis is suspected.

(2) Incubate SF medium for 18-24 hours at 37º C. This medium is specific for the growth and reproduction of Streptococcus faecalis.

1-20. COLLECTING AND PROCESSING BODY FLUIDS

NOTE: See figure 1-10.

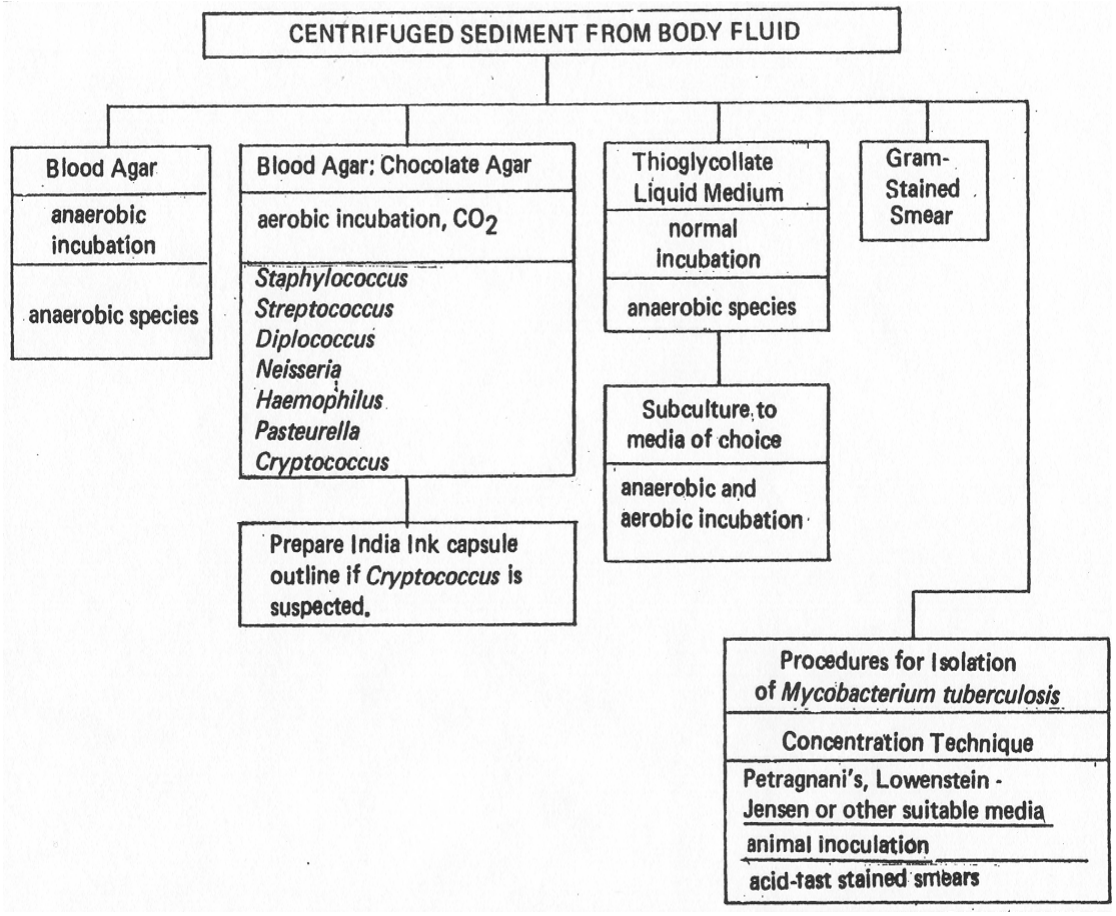


Figure 1-10. Technique for processing body fluids.

a. **Importance.** Body fluids include spinal, synovial, pleural, pericardial, and peritoneal fluid. Examination of spinal fluids is important in determining the cause of meningitis. In many cases, emergency therapeutic agents are administered on the basis of results from microscopic examination (gram-stained smear) of concentrated spinal fluid. The genera of microorganisms most frequently responsible for meningitis are Neisseria, Diplococcus, Streptococcus, Staphylococcus, Haemophilus, and occasionally Mycobacterium. One of the fungi chiefly responsible is Cryptococcus neoformans. Neisseria may be encountered in specimens of synovial fluid from arthritic patients.

b. **Collection.** When a medical officer submits a body fluid for culture, the source and the provisional diagnosis should be entered on the request form. This will aid in the selection of the proper media for inoculation. Generally, all body fluids are aspirated by a medical officer and transported to the laboratory in sterile, screw-capped test tubes. Culture body fluids as soon as possible after collection since fibrinogen coagulates the fluids, making them difficult to transfer. Use extreme care when handling body fluids because these fluids often contain highly infectious organisms.

c. **Culture for Routine Organisms.**

(1) Mix the specimen well and centrifuge at 2,500 rpm for approximately 15 minutes.

(2) Decant the supernatant into a jar of phenol. Inoculate a portion of the concentrated sediment to two blood agar plates, one supplemented chocolate agar plate, and one tube of thioglycollate liquid medium.

(3) Prepare a direct smear from the sediment: gram stain and observe for predominating organisms.

(4) Incubate one blood agar plate and the chocolate agar plate under CO_2 tension for 18-24 hours at 37º C.

(5) Incubate the second blood agar plate anaerobically for 18-24 hours at 37º C.

(6) Incubate the thioglycollate liquid medium under normal incubation atmosphere for 18-24 hours at 37º C.

(7) If growth is observed in the thioglycollate liquid medium, subculture this growth under anaerobic conditions and aerobic conditions.

d. **Culture for Acid-Fast Bacilli.**

(1) The sediment from the centrifuged specimen need not be further concentrated, providing the specimen is free from mucus, debris, and contaminating growth.

(2) The processed sediment may be inoculated to suitable media, injected into test animals, and/or spread on clean slides and dried for acid-fast staining. Lowenstein-Jensen or Petragnani's medium is satisfactory for culturing acid-fast organisms.

1-21. COLLECTING AND PROCESSING EXUDATE SPECIMENS

NOTE: See figure 1-11.

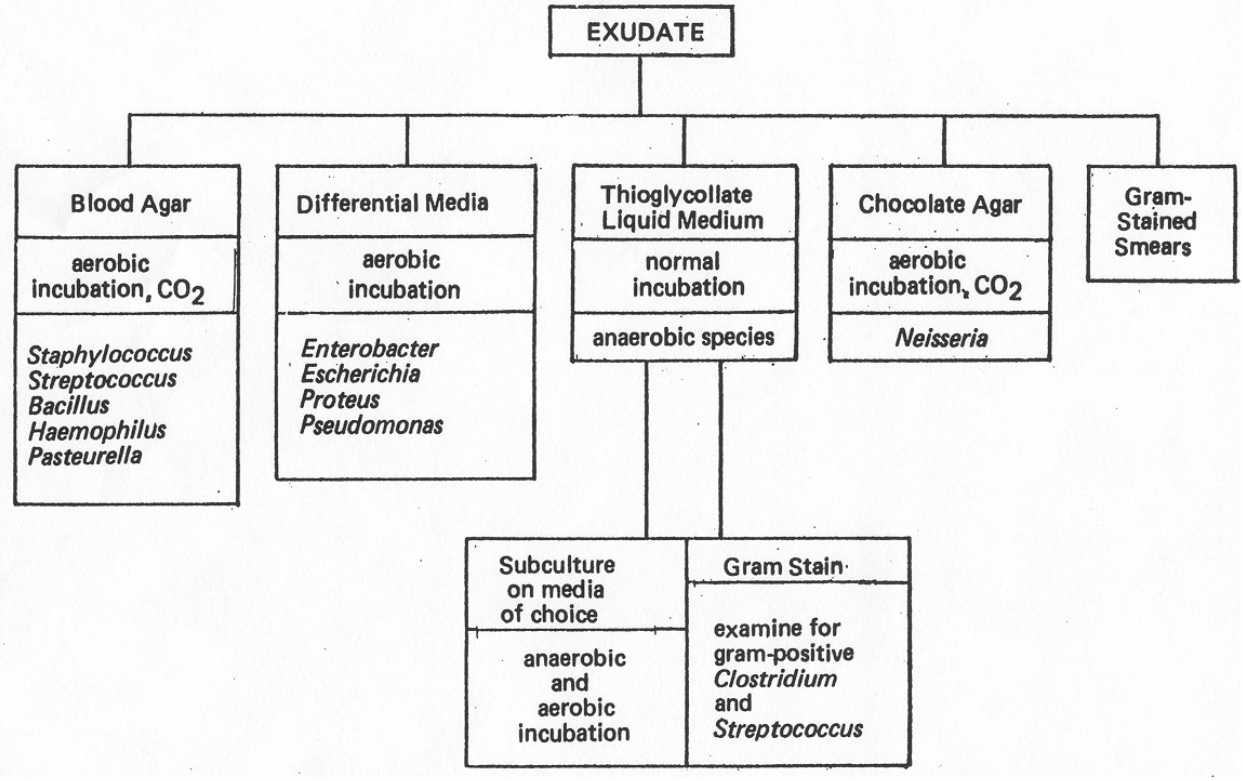


Figure 1-11. Technique for processing exudates.

a. **Importance.** An exudate is material that has passed through the walls of vessels into adjacent tissues or areas of inflammation. Exudates may be obtained from boils, wounds, ear or mastoid infections, eye infections, and skin lesions. A great variety of microorganisms may be isolated from such areas. Boils may yield Staphylococcus species, Streptococcus species, and occasionally gram-negative rods (Escherichia, Pseudomonas, or Proteus). Deep wounds, especially puncture wounds, and those with severe tissue damage provide ideal living conditions for anaerobes, especially Clostridium species. Infected wounds that exhibit a greenish or bluish purulent discharge may reveal Pseudomonas infection. Exudates from surgical or postoperative infections may reveal anaerobic Streptococcus species. . Ear and mastoid infections are often caused by Pseudomonas, Staphylococcus, or Streptococcus species. Eye infections may yield Haemophilus or possibly Neisseria species. Purulent discharges from the urethra frequently reveal Neisseria gonorrhea. A gram-stained smear of exudate from chancroid or soft chance usually reveals small gram-negative rods (Haemophils ducreyi). A dark field examination of exudate from the true or hard change usually reveals the spirochete Treponema pallidum.

b. **Collection.** Exudate specimens are usually collected on sterile cotton-tipped swabs by a medical officer and sent to the laboratory in sterile cotton-plugged test tubes. Exudate material in the form of purulent drippings from cases of urethritis may be collected on sterile cotton-tipped swabs or on sterile wire loops and inoculated directly on or into culture media.

c. **Culture.**

(1) Inoculate the specimen on blood agar, EMS or MacConkey's and thioglycollate liquid medium.

(2) If Neisseria is suspected, inoculate a supplemented chocolate agar plate.

(3) Prepare direct smears, gram-stain, and examine microscopically for predominating organisms.

(4) Incubate the media for 18-24 hours at 37º C. Place the blood and chocolate agar plates under CO_2 tension for the period of incubation.

(5) If growth is observed in the thiolycollate liquid medium, this growth under anaerobic conditions and aerobic conditions.

(6) Prepare gram-stained smears from any growth in the thioglycollate medium.

1-22. PROCESSING OF SPECIMENS FOR SHIPMENT

NOTE: See table 1-2.

It is frequently necessary to ship specimens to reference laboratories for analysis or confirmation. Acceptable shipping containers and procedures for packing specimens are described below:

SPECIMEN	PROCESSING FOR SHIPMENT	REMARKS
Ascitic fluid for bacterial and fungal culture.	Place aseptically in sterile containers without preservative.	Where tuberculosis is suspected, see directions for processing specimens from suspected tuberculosis patients.
Pure cultures of bacteria for identification, confirmation, and sensitivity tests.	Grow in pure culture on beef infusion agar in screw-cap tubes. Prior to shipment, tighten screw-caps and secure with adhesive tape or by dipping in melted paraffin.	Plant subcultures on agar slants and incubate 12-18 hours. A duplicate of each culture should be retained in the laboratory of origin until a final report is rendered.
Blood or urine for culture.	Collect 3 ml. of blood aseptically before serum, chemical or antibiotic therapy and transfer to rubber-stoppered bottle containing hemoglobin-tryptosephosphate broth.	Prior to shipment, incubate specimens for 12-24 hours.
Serum for bacterial and cold agglutinins, antibiotic levels, and complement-fixation tests.	Collect blood aseptically in sterile evacuated tubes, filling to capacity.	Allow firm clot to form before packing for shipment. Blood for cold agglutination tests must not be refrigerated until cells are separated from serum.

Table 1-2. Collection and processing of bacteriological specimens for shipment (continued).

SPECIMEN	PROCESSING FOR SHIPMENT	REMARKS
Cerebrospinal fluid for bacterial culture, and antibiotic and sulfonamide level.	A minimum of 3 ml. of fluid is collected aseptically and inoculated into hemoglobin-tryptosephosphate broth.	Where tuberculosis is suspected, see directions for processing such specimens. Retain broth culture under incubation 12-24 hours prior to shipment.
Throat cultures for *C. dipheriae*.	Innoculate Loeffler's serum slant with a fresh throat swab.	Mail specimen immediately.
Joint or other body fluids or bacterial and fungal cultures.	Collect joint and other body fluids and transfer aseptically to suitable sized sterile container without preservative. Plant fluids for mycological culture on Sabouraud's media, or place in sterile screw-capped containers without preservative.	
Sputum for pneumococcus culture or typing.	Collect aseptically before serum, chemical, or antibiotic therapy is started and place in sterile container without preservative.	
Swabs for culture from open lesions.	Swab lesion with broth-soaked swab and place aseptically in sterile screw-capped test tubes.	
Tuberculosis, specimens for smear, culture, and animal inoculation (other than sputum and gastric contents).	Collect fluids, tissue, or feces in suitable sized sterile containers without preservative.	Specimens include ascitic fluid, cerebrospinal fluid, joint fluid, pleural fluid, pus, urine, lymph node, and other tissue, and feces.

Table 1-2. Collection and processing of bacteriological specimens for shipment (continued).

SPECIMEN	PROCESSING FOR SHIPMENT	REMARKS
Tuberculosis, sputum specimens.	Collect all sputum coughed up on three consecutive mornings after first having patient wash teeth and rinse mouth thoroughly with boiled water. Place entire 3-day specimen in a sterile wide-mouthed screw-capped bottle; add an equal volume of 20% trisodium phosphate.	During collection, keep jars out of direct sunlight or the tubercle bacillus will be rendered nonviable and, therefore, unsuitable for culture or animal inoculation. Do not use cardboard sputum cups for collection.
Tuberculosis, gastric contents.	Through sterile stomach lavage tube introduce 200-300 ml. of sterile saline solution into fasting stomach. Evacuate stomach and place entire specimen in suitable sterile container.	Prior to placing specimen in shipping container, neutralize it to litmus with 20 percent sodium bicarbonate.
Exudates for bacterial and fungal cultures.	Place swabs aseptically in sterile screw-capped containers without preservative.	
Fecal specimens for bacterial culture (except tuberculosis).	Emulsify a 5 g. sample, aseptically in a sterile screw-capped vessel containing one part C.P. glycerin and three parts 0.6% saline solution.	In food poisoning outbreaks, feces should be collected from patients with diarrhea and all food handlers associated with the outbreak.

Table 1-2. Collection and processing of bacteriological specimens for shipment (continued).

SPECIMEN	PROCESSING FOR SHIPMENT	REMARKS
Specimens for bacteriological examination following food poisoning outbreaks.	(1) Any suspected canned foods should be shipped unopened in original container. (2) Well mixed representative samples of suspected prepared food should be placed aseptically in suitable containers without preservative. (3) Vomitus from all cases in which vomiting is present should be placed aseptically in suitable sized sterile containers without preservative. (4) Swabs from lesion taken from food handlers should be placed in nutrient broth using aseptic technique and transferred to sterile screw-capped test tubes.	Complete epidemiological history should accompany any specimens taken from food poisoning outbreaks.

Table 1-2. Collection and processing of bacteriological specimens for shipment (concluded).

a. **Noninfectious Specimen.**

(1) Enclose the specimen in a sterile glass container and cap. A screw-shaped test tube or jar is satisfactory.

(2) Place the glass container in standard double shipping containers.

(3) Pad the spaces between the containers to guard against breakage.

(4) The properly packed container of noninfectious material will have the following layers, from specimen outward: specimen, glass, padding, metal, padding, and heavy cardboard (figure 1-12A).

b. **Infectious Specimen.**

(1) Enclose the specimen in a sterile, stout glass tube and seal the ends of the tube by fusion of the glass.

(2) Place the glass tube containing the specimen in a stout glass container that can be sealed by an insulated screw cap, rubber stopper, or by fusion of the glass.

(3) Add formalin to the outer container so that the inner glass container is completely surrounded by formalin: then seal the glass container with wax. This procedure provides for disinfection if the inner container should break.

(4) Pack this double glass container in standard double shipping containers.

(5) Pad the spaces between the containers to guard against breakage.

(6) The properly packed container of infectious material will have the following layers, from specimen outward: specimen, glass, formalin, glass, padding, metal, padding, and heavy cardboard (figure 1-12B).

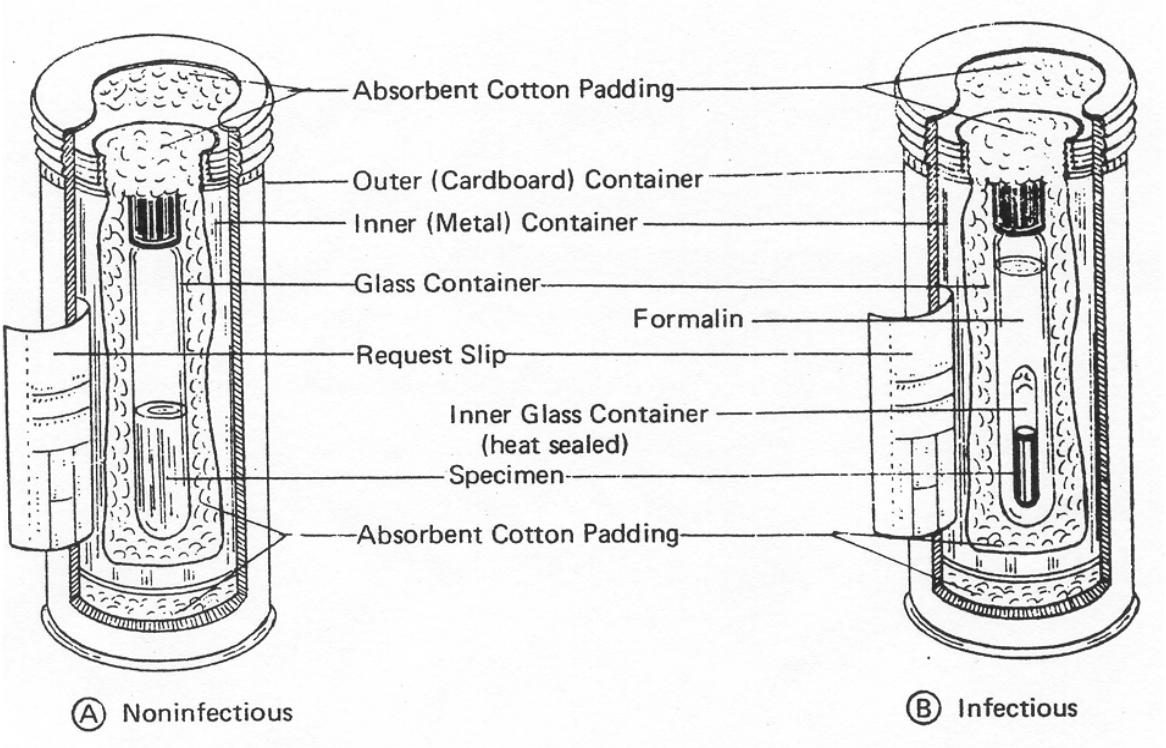


Figure 1-12. Microbiological specimens packed for shipment.

c. **Labeling.**

(1) The appropriate request form, properly completed, must accompany the specimen.

(2) The request slips should be placed between the two outer shipping containers to prevent the slips from being damaged or contaminated if breakage occurs.

(3) The following notation must be made on the outside label of any mailing container of bacteriological specimens: Specimen for bacteriological examination; this package shall be packaged with letter mail.

Continue with Exercises

EXERCISES, LESSON 1

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. Mutualism is a microbial association in which:

   a. The host and microorganism both benefit.

   b. The microorganism derives significant benefit at the expense of the host.

   c. Neither the microorganism nor the host derives any benefit, or harms the other.

   d. The host derives significant benefit at the expense of the microorganism.

2. Commensalism is a microbial association in which:

   a. The host and microorganism both benefit.

   b. The microorganism derives significant benefit at the expense of the host.

   c. The microorganism derives significant benefit without harming or greatly benefiting the host.

   d. The host derives significant benefit at the expense of the microorganism.

3. The LD_{50} is a standardized means of expressing:

   a. Invasiveness.

   b. Virulence.

   c. Pathogenesis.

   d. Infectiousness.

4. Bacteria commonly reproduce by:

    a. Spore germination.

    b. Conjugation.

    c. Budding.

    d. Binary fission.

5. A toxin that is secreted by bacteria into their environment is known as:

    a. An exotoxin.

    b. An endotoxin.

    c. A fomite.

    d. A proteolytic enzyme.

6. A toxin that is found in the cell wall of a living organism is:

    a. An endotoxin.

    b. An enterotoxin.

    c. An exotoxin.

    d. A pseudotoxin

7. Bacteria that is able to obtain energy and grow on inorganic media and utilize carbon dioxide as their sole source of carbon are known as:

    a. Heterotrophic bacteria.

    b. Autotrophic bacteria.

    c. Parasitic bacteria.

    d. Saprophytic bacteria.

8. Heterotrophic bacteria require _____ media.

    a. Organic.

    b. Inorganic.

9. Bacteria are measured in microns, which are:

    a. 1/10 of a millimeter.

    b. 1/100 of a millimeter.

    c. 1/1,000 of a millimeter.

    d. 3.1416 parts of an inch.

10. Spherical bacteria arranged in chains are called:

    a. Spirilla.

    b. Diplococci.

    c. Streptococci.

    d. Staphylococci.

11. The outer layer that gives rigidity to a bacterial cell is known as the:

    a. Cell wall.

    b. Cytoplasmic membrane.

    c. Nuclear membrane.

    d. Cytoplasm.

12. Flagella are:

    a. Gummy envelopes surrounding organisms.

    b. Internal structures formed for protection.

    c. Thread-like appendages for locomotion.

13. One would expect to find spores in:

    a. ALL OF THE BELOW.

    b. Cocci.

    c. Bacilli.

    d. Spirilla.

14. The formation of spores by bacteria is generally considered as a means of:

    a. Reproduction.

    b. Toxin formation.

    c. Survival.

    d. Resistance to phagocytosis.

15. Two media suitable for initial culture of blood specimens are:

    a. EMB agar and MacConkey's agar.

    b. Thioglycollate broth and Trypticase soy broth.

    c. Brilliant green agar and bismuth sulfite agar.

    d. Deoxycholate agar and Salmonella-Shigella agar

16. Blood cultures should be observed daily. They should be checked for the presence of bacterial growth at the first sign of:

    a. ANY OF THE BELOW.

    b. Turbidity.

    c. Gas formation.

    d. Coagulum formation.

17. Blood cultures should be observed for at least _____ before being reported as negative and discarded.

    a. 1 week.

    b. 2 weeks.

    c. 3 weeks.

    d. 4 weeks.

18. Normal throat cultures usually show a predominance of

    a. Alpha hemolytic streptococci and neisseriae.

    b. Corynebacteria.

    c. Bordetella.

    d. Beta hemolytic streptococci.

19. If diphtheria is suspected, a throat culture should be inoculated on which of the following special media in addition to those used routinely?

    a. Chocolate agar.

    b. Potassium tellurite agar.

    c. Petragnani's agar.

    d. Lowenstein-Jensen agar.

20. The disk is used as a screening test for:

    a. Pneumococci.

    b. Streptococci.

    c. Staphylococci.

    d. Corynebacteria.

21. The generally preferred method of urine collection is:

    a. Catheterization.

    b. 24-hour collection.

    c. Two-container collection.

    d. The clean-catch method.

22. When urine specimens cannot be processed within 1 hour of collection, they must be stored at what temperature?

    a. Below 0° C.

    b. 4° C.

    c. 25° C.

    d. 37º C.

23. For the initial suppression of most enteric saprophytes in stool specimens being cultured for enteric pathogens, which of the following should be used?

    a. Blood agar.

    b. Nutrient broth.

    c. Trypticase soy agar.

    d. Selenite F broth.

24. A subculture on bismuth sulfite agar of suspected Salmonella typhi organisms should be incubated at 37º C for how long before being discarded as negative:

    a. 12 hours.

    b. 24 hours.

    c. 48 hours.

    d. 72 hours.

25. SF medium is specific for the growth and reproduction of:

    a. Sarcophaga fuscicauda.

    b. Shigella flexneri.

    c. Siphunculina funicola

    d. Streptococcus faecalis.

26. Examination of spinal fluids is important in determining the cause of:

    a. Meningitis.

    b. Pleurisy.

    c. Pneumonia.

    d. Urethritis.

27. Which of the following would be considered an exudate rather than a body fluid?

    a. Synovial fluid.

    b. Pericardial fluid.

    c. Peritoneal fluid.

    d. Purulent discharge from a wound.

28. Which of the following would be considered an exudate?

    a. Fluid from the peritoneum.

    b. Fluid from the pericardium.

    c. Fluid from the joints.

    d. Fluid from a boil.

29. If Neisseria is suspected in exudate material, the specimen should be plated on blood agar, EMB, thioglycollates and:

    a. Brilliant green agar.

    b. Chocolate agar.

    c. Selenite F broth.

    d. Tetrathionate broth.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES, LESSON 1

1. a (para 1-1a)
2. c (para 1-1b)
3. b (para 1-2b)
4. d (para 1-2d)
5. a (para 1-5a)
6. a (para 1-5b)
7. b (para 1-9)
8. a (para 1-10)
9. c (para 1-11; Figure1-1)
10. c (para 1-11a(3))
11. a (para 1-12a(1))
12. c (para 1-12b(2))
13. c (para 1-12b(3))
14. c (para 1-12b(3))
15. b (para 1-16f, g)
16. a (para 1-16i)
17. c (para 1-16i)
18. a (para 1-17c(2))
19. b (para 1-17c(5))
20. a (para 1-17d(1))
21. d (para 1-18b)
22. b (para 1-18b)

23. d (para 1-19c(1))

24. c (para 1-19c(5))

25. d (para 1-19d (2))

26. a (para 1-20a)

27. d (para 1-21a)

28. d (para 1-21a)

29. b (para 1-21c(2))

End of Lesson 1

LESSON ASSIGNMENT

LESSON 2 Microscopic Examination and Cultivation of Bacteria.

TEXT ASSIGNMENT Paragraphs 2-1 through to 2-39**.**

LESSON OBJECTIVES After completing this lesson, you should be able to:

2-1. Identify the proper methods of preparing smears for staining and preparing wet unstained mounts.

2-2. Identify the principle, re- agents, order of steps, methods of interpreting results, significance, and sources of error for the:
   a. Acid-fast stain (Ziehl-Neelsen).
   b. Acid-fast stain (Kinyoun modified.
   c. Capsule outline (India ink).
   d. Capsule stain (Hiss method).
   e. Flagella stain (Leifson method modified).
   f. Gram stain (Hucker's modification).
   g. Methylene blue stain.
   h. Spore stain (Wirtz-Conklin).

2-3. Identify the common components and ph of media and identify their functions.

2-4. Identify appropriate environmental factors for bacterial growth and associate related terms with their correct definitions.

2-5. Identify correct applications of anaerobic methods.

2-6. Associate the terms bactericidal and bacteriostatic with their correct definitions.

2-7. Associate alcohol and phenol with their uses.

2-8. Identify proper methods and effects of sterilization of common laboratory materials.

2-9. Identify correct applications of aseptic technique.

2-10. Identify the purpose and the current applications of the streak plate method and pour plate method.

SUGGESTION After reading and studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 2

MICROSCOPIC EXAMINATION AND CULTIVATION OF BACTERIA

Section I. MICROSCOPIC EXAMINATION OF BACTERIA

2-1. GENERAL COMMENTS

The direct observation of microorganisms in stained or unstained slide mounts of cultures or specimens is an essential part of clinical bacteriology. Characteristics such as the shape and grouping of the cells, the presence or absence of special structures, and the reaction to stains, are of value in identification.

2-2. PREPARATION OF SMEARS FOR STAINING

a. **Smears Made From Clinical Specimens.**

(1) If clinical materials such as pus or similar exudates are to be examined, liberal samples should be spread to a thin film over a rather large area on a clean glass slide. This is successfully accomplished using a sterile wire loop.

(2) When paired swab specimens are submitted, the material on one swab may be smeared directly on the slide. Smears of swabs containing dried material (undesirable) or meager amounts of specimen are best prepared by emulsification in a drop of normal saline on the slide.

(3) When preparing smears of clinical specimens such as sputum or feces, care should be taken to select any flecks of mucus or blood-tinged particles present, since they are more likely to yield infectious agents on microscopic examination.

(4) In many instances, the detection of etiological agents in fluid specimens (urine, spinal fluid, etc.) may be accomplished only by observing stained smears of concentrated sediments following centrifugation. Smears of concentrated specimens are conveniently prepared using a sterile, rubber-bulbed capillary pipet. After pipetting 3 or 4 drops of the concentrate on a slide, a sterile wire loop or clean wooden applicator stick may be used to spread the material to an even film of proper density.

(5) As a general rule, smears of clinical materials must be thicker than smears of bacterial growth from liquid or solid culture media. It is highly desirable that more than one film of a given specimen be prepared. This will increase the probability of observing etiologic agents.

(6) It should always be remembered that if necessary, a smear may be made from the same swab that has been used to culture a specimen. However, the culture must always be prepared first, followed by the slide. Never use a swab to make a slide and then use the same swab to plate a culture because you will contaminate your culture.

b. **Smears Made From Liquid (Broth) Specimens.** When fluid cultures are to be examined, smears are prepared by taking a loopful of the medium and spreading it on a glass slide. If the broth is highly turbid, the droplet should be spread over an area somewhat smaller than the size of a dime. When growth is meager, it may be necessary to allow 2 or 3 loopfuls of medium to dry in a concentrated film on the slide.

c. **Smears Made From Growth on Solid Media.** If smears are to be prepared from a culture on solid media, a minute amount of growth is picked from a pure colony and emulsified in a droplet of fresh (bacteria-free) water on a glass slide. This is best accomplished using a straight needle or loop. The suspension of organisms should be only slightly cloudy. Otherwise, the smear will be too thick for microscopic observation of individual cells.

d. **Preparation for Staining.** All smears should be allowed to air dry. They are then heat-fixed by passing the slide back and forth through a gas flame 3 or 4 times. When the slide has cooled, the smear is ready for staining. Smears must be quickly passed through the flame and not held in the flame, as this will scorch the organisms and destroy their morphological characteristics.

e. **Use of Stains.** Depending upon the nature of the particular bacteriologic study, smears may be stained by anyone of the methods listed below. In some instances, smear preparation must be varied to suit the needs of the staining method used, e.g., capsule or flagella stains.

2-3. ACID-FAST STAIN (ZIEHL-NEELSEN)

a. **Principle.** Members of the genus Mycobacterium and certain Nocardia species possess relatively large amounts of lipids, fatty acids, and waxes within their cell. These substances resist penetration and staining of the cytoplasm by ordinary methods. When these organisms are exposed to a high concentration of basic dye and heat is applied, staining is more readily accomplished. Once stained, the organisms will resist decolorization with acid-alcohol even upon prolonged exposure. Although all bacteria may be stained in this manner, only the above organisms, with rare exceptions, will resist decolorization with the acid-alcohol solution.

b. **Reagents.**

(1) Carbolfuchsin stain (Ziehl-Neelsen). Dissolve 1 9 *of* basic fuchsin in 10 ml of 95 percent ethyl alcohol. Add sufficient 5 percent phenol (aqueous solution) to make 100 ml.

(2) Acid-alcohol. Using a transfer pipet, place 3 ml of concentrated hydrochloric acid (HCl) in a 100-ml volumetric flask. Add enough 95 percent ethyl alcohol to make 100 ml.

(3) Counterstain. Dissolve 0.3 g of methylene blue in 30 ml of 95 percent ethyl alcohol. Add enough distilled water to make 100 ml.

c. **Technique.**

(1) Flood the heat-fixed smear with carbolfuchsin stain and gently steam the preparation for 3-5 minutes by passing a flame under the flooded smear. Do not allow the stain to boil or dry on the slide.

(2) Remove excess stain with a gentle flow of water.

(3) Holding the slide at an angle, decolorize by dropping acid-alcohol over the smear until the solution flows colorless.

(4) Remove excess decolorizer with a gentle flow of water.

(5) Apply the methylene blue counterstain for 1 minute.

(6) Remove excess counterstain with a gentle flow of water. Blot dry.

(7) Examine the slide using the oil immersion objective.

d. **Interpretation.**

(1) Organisms which retain the primary stain (carbolfuchsin), after the smear is treated with acid-alcohol decolorizer, appear pink to red. They are, therefore, termed acid fast.

(2) All non-acid-fast organisms are readily decolorized with acid-alcohol: consequently, they stain blue with the counterstain.

e. **Discussion.** The Ziehl-Neelsen or modified Kinyoun techniques (see below) are routinely used in examining specimens and/or cultures for the presence of the acid-fast tubercle bacilli. The particular procedure used is determined by the preference of the individual worker. In the Ziehl-Neelsen technique, time and temperature of heating are somewhat critical. Overheating diminishes the color of the organisms to a pink or brown or may even distort the shape of the cell.

2-4. ACID-FAST STAIN (KINYOUN MODIFIED)

a. **Principle.** The Kinyoun technique is similar to the Ziehl-Neelsen technique except that a wetting agent, instead of heat, is used to facilitate staining.

b. **Reagents.**

(1) Carbolfuchsin stain (Kinyoun). Dissolve 4 g of basic fuchsin in 20 ml of 95% ethyl alcohol. While shaking the mixture, Slowly add 100 ml of distilled water. Melt concentrated phenol in a 560^{O} C water bath and pipet 8 ml of phenol into the mixture.

(2) Tergitol No.7. Available commercially.

(3) Acid-alcohol. See under reagents for Ziehl-Heelsen method above.

(4) Counterstain. See under reagents for Ziehl-Neelsen method above.

c. **Technique.**

(1) Flood the heat-fixed smear with carbolfuchsin stain.

(2) Add 1 drop of the wetting agent, Tergitol No.7, to the stain-covered smear and allow the mixture to stand for 1 minute (no heat is necessary).

(3) Was the slide thoroughly with water.

(4) Continue with step (5) of the Ziehl-Neelsen acid-fast stain.

d. **Interpretation.** Smears stained by this method are interpreted in the same manner as smears stained using the Ziehl-Neelsen technique.

e. **Discussion.** Tergitol reduces the surface tension between the cell wall of acid-fast organisms and the carbolfuchsin stain, as does heat in the Ziehl-Neelsen method. The heatless Tergitol method is more advantageous in that-it is less cumbersome and requires less time.

2-5. CAPSULE OUTLINE (INDIA INK)

a. **Principle.** India ink is not absorbed by the cells or capsules of organisms. When encapsulated forms are mixed -with India ink on a glass slide, the dark background of ink particles clearly outlines the colorless capsule surrounding the more dense appearing, centrally situated cell.

b. **Reagent.** India ink preserved with 0.5 percent phenol.

c. **Technique.**

(1) Place a loopful of dilute specimen (broth culture or normal saline suspension) on a clean glass slide.

(2) Place a loopful of India ink on the slide adjacent to the specimen.

(3) Join the two droplets with a wire loop without mixing.

(4) Gently add a cover slip, allowing the droplets to infuse by capillary action.

(5) Examine the preparation immediately using the oil immersion lens, reducing the light with the diaphragm until the bacterial cells are clearly in focus.

d. **Interpretation.** The capsules, when present, appear as clear halos around the bacteria.

e. **Discussion.**

(1) Not all brands of India ink are satisfactory for demonstrating capsules. Inks should be relatively free of bacterial contamination and must not contain any encapsulated organisms. Suitable inks may be protected against contamination by adding phenol to secure a 0.5 percent concentration by volume.

(2) Inks containing very finely divided carbon particles yield the best results.

(3) Capsules are best demonstrated in young cultures (4-6 hours), however, this is largely determined by the growth rate of the organism concerned.

2-6. CAPSULE STAIN (HISS METHOD)

a. **Principle.** Bacterial capsules are composed of polysaccharide compounds which do not stain well by ordinary method. Encapsulated bacteria exhibit capsular swelling when mixed with normal serum. Capsules may be stained after impregnation with serum. This is the basis of the Hiss technique.

b. **Reagents.**

(1) 1% crystal violet. Dissolve 1 g of crystal violet in less than 100 ml of distilled water. Add sufficient distilled water to make 100 ml.

(2) 20% copper sulfate. Dissolve 20 g of copper sulfate ($CuSO_4$, chemically pure) in 50-75 ml of distilled water. (It is easier to dissolve the copper sulfate if it is ground with a mortar and pestle prior to weighing.) Add sufficient distilled water to make 100 ml.

(3) Normal serum (rabbit or human).

c. **Technique.**

(1) Mix a loopful of specimen, broth culture, or normal saline-suspended growth with a drop of normal serum on a glass slide. Spread the mixture.

(2) Allow the smear to air-dry at room temperature.

(3) With the film side up, heat-fix the preparation passing it three or four times through a flame. Do not overheat.

(4) Cover the smear with crystal violet stain.

(5) Steam the preparation gently over a flame for 30 seconds to 1 minute.

(6) Wash off the stain gently with copper sulfate solution.

(7) Gently blot off the excess solution and set the slide aside to dry at room temperature.

(8) Examine the preparation using the oil immersion objective.

d. **Interpretation.** With this method, the capsules appear as faint blue halos around dark blue to purple cells.

2-7. FLAGELLA STAIN (LEIFSON METHOD MODIFIED)

a. **Principle.** A flagella-staining solution is prepared to contain a basic dye along with a substance composed of fine particulate matter in collodial suspension. Upon adding the stain to a dried smear of motile bacteria, the dye-covered particles are deposited on the flagellar filaments rendering them microscopically visible.

b. **Reagents.**

(1) Flagellar stain. Dissolve 1.7 g of basic fuchsin-tannic acid dye in 35 ml of 95 percent ethyl alcohol. Add enough distilled water to make 100 ml.

(2) Counterstain. Dissolve 0.1 g *of* methylene blue and 1.0 g of borax in less than 100 ml of distilled water. Add enough distilled water to make 100 ml.

c. **Technique.**

(1) Suspend a small amount of growth from agar medium in distilled water or normal saline to obtain a slightly turbid suspension.

(2) Place a large loopful of suspended organism on a clean glass slide.

(3) Tilt the slide lengthwise and allow the suspension to run down to form a film.

(4) Allow the film to air-dry; do not heat-fix.

(5) Flood the smear with the flagellar stain for 10 minutes.

(6) Wash the slide thoroughly with water. Counterstain for 5 mi to 10 minutes.

(7) Wash the slide, blot dry, and examine using the oil immersion lens.

d. **Interpretation.** The flagella of various bacteria appear as extremely slender, fragile filaments that may be spiraled.

e. **Discussion.** The use of scrupulously clean, new slides and bacterial suspensions free of foreign organic matter is necessary in order to obtain successful staining of flagella.

2-8. GRAM STAIN (HUCKER'S MODIFICATION)

The most widely used staining procedure in the bacteriological laboratory is the gram stain. The purpose of the gram stain is to differentiate bacteria on the basis of their gram-staining reaction.

a. **Principle.** Gram-positive bacteria, following initial staining with crystal violet, will retain the purple dye upon subsequent treatment with a mordant (iodine) and the application of alcohol or acetone-alcohol decolorizing agents. Gram-negative bacteria, which lack specific cellular substances responsible for binding crystal violet, fail to retain the dye upon similar treatment. The latter forms are, therefore, stained red upon application of safranin counterstain.

b. **Reagents.**

(1) Crystal violet stain.

(a) Make SOLUTION A by dissolving 4 g of crystal violet in 20 ml of 95 percent ethyl alcohol.

(b) Make SOLUTION B by dissolving 0.8 g of ammonium oxalate [$(NH_4)_2C_2O_4 \cdot H_2O$] in 80 ml of distilled water.

(c) Mix solutions A and B ordinarily in equal parts.

(d) This procedure may sometimes cause organisms such as Gonococcus to retain the basic dye and resist decolorization. To avoid this, solution A may be diluted as much as 10 times. Then the diluted solution A is added in equal parts to solution B.

(2) Iodine solution.

(a) Grind 1 g of iodine (I_2) and 2 g of potassium iodide (KI) in a mortar. Dissolve the ground reagents in 5 ml of distilled water and add sufficient distilled water to make 240 ml. Add 60 ml of 5 percent sodium bicarbonate (made by adding enough distilled water to 5 g of sodium bicarbonate ($NaHCO_3$) to make 100 ml).

(b) When a color loss of the iodine solution is noted, prepare a fresh solution. The iodine solution will remain stable longer if stored in a dark bottle.

(3) Decolorizer. Mix equal volumes of 95 percent ethyl alcohol and acetone.

(4) Safranin counterstain. Dissolve 0.5 g of safranin in a small amount of distilled water. Add sufficient distilled water to make 100 ml.

c. **Technique.**

(1) Cover the heat-fixed smear with crystal violet solution, allowing the stain to remain for 1 minute.

(2) Remove all stain with a gentle flow of water.

(3) Cover the smear with iodine solution (mordant) for at least 1 minute.

(4) Wash with water.

(5) Treat with decolorizer solution by flowing the reagent drop wise over the smear while the slide is held at an angle. Decolorization should be stopped as soon as the drippings from the slide become clear.

(6) Remove excess decolorize immediately with a gentle flow of water.

(7) Apply the safranin counterstain for 30 seconds.

(8) Remove counterstain with gentle flow of water and gently blot dry.

(9) Examine the smear microscopically using the oil immersion objective.

d. **Interpretation.**

(1) Most bacteria may be placed in one of two groups by their reaction to the gram stain. If an organism retains crystal violet (cells purple or blue), it is referred to as gram-positive. Organisms that lose crystal violet stain under treatment with the decolorizing agent and are stained red upon applying the safranin counterstain are termed gram-negative.

(2) Gram-positive organisms may become gram-negative as a result of autolysis, aging, acidity of culture medium, improper temperature of incubation, or the presence of toxic substances (drugs, metabolic wastes, etc). For best results, gram stains should be prepared on cultures 18-24 hours old. A known gram-positive organism may be used as a control.

(3) If films are prepared unevenly, or excessively thick, dense clumps of growth will be present which retain crystal violet upon decolorization, regardless o-f the gram reaction. Under these conditions, gram-positive appearing clumps may be present in an otherwise gram-negative smear.

(4) Smears should be completely dry before being heat-fixed; otherwise, any protein material carried over into the smear from culture media or specimens will be precipitated. As a result the background of the smear will be difficult to decolorize and may be filled with debris and misleading artifacts.

(5) False decolorization of gram-positive cells may result from the use of an iodine (mordant) solution that has deteriorated. Gram's iodine solution will remain stable for longer periods of time when protected from light by storage in a dark colored bottle. When the iodine (mordant) solution fades from a dark brown to light amber or yellow, it is no longer suitable for use.

(6) Too drastic a treatment with the decolorizing solution usually results in a false gram-negative reaction. Immediately after the drippings appear clear, when treating the smear with acetone-alcohol mixture, the slide must be washed with water to prevent over- decolorization.

(7) Although most bacteria are either gram-positive or gram- negative, certain species exhibit a definite tendency to display both gram-positive and gram-negative forms ("gram-variable"). Usually whether positive, negative, or variable the gram reaction is species specific.

e. **Discussion.**

(1) The gram stain is extremely valuable as a screening technique for differentiating bacterial types. By correlating the gram reaction with the basic morphology exhibited by a particular organism in a gram-stained smear, tentative generic identification is often accomplished. This enables intelligent selection of suitable culture media for isolation and confirmation of the organism involved. Furthermore, the gram reaction is often concomitant with other properties of a given group or species of bacteria, e.g., habitat, resistance or susceptibility to therapeutic agents, disease manifestations, etc.

(2) The gram stain is routinely employed as the first step in the examination of specimens and cultures. The necessity for a laboratory technician to be able to successfully prepare and interpret gram-stained smears cannot be overemphasized.

2-9. METHYLENE BLUE STAIN

a. **Principle.** Methylene blue stain is used to impart a purple color to metachromatic granules and a light blue color to the remainder of each cell.

b. **Reagent.** Dissolve 0.5 g methylene blue in 30 ml of 95 percent ethyl alcohol. Add enough distilled water to make 100 ml.

c. **Technique.**

(1) Cover the heat-fixed smear with the staining solution for 1-2 minutes.

(2) Wash the slide thoroughly with water, then gently blot it out.

(3) Examine the preparation using the oil immersion objective.

d. **Interpretation.**

(1) In species of bacteria containing metachromatic granules, the latter will stain intensely purple giving the cells a beaded, barred, or banded appearance.

(2) The dark purple stained granules stand out clearly against the cytoplasm that stains pale blue.

e. **Discussion.** Methylene blue is commonly used for staining smears f throat specimens or cultures thereof, for the presence of Corynebacterium diphtheriae.

2-10. SPORE STAIN (WIRTZ-CONKLIN)

a. **Principle.** Bacterial spores are difficult to stain. In smears stained by ordinary methods, they appear as unstained gaps or holes within the cytoplasm of bacillary cells. Free spores may appear as tiny blue, brown, or red rings, for only the outer shell of the spore is faintly stained. Bacterial spores are more readily demonstrated by special staining methods that consist of steaming the dye-covered smear on a glass slide for a brief period. Staining is effected by lowering the surface tension between the spore coat and dye solution. Once the spore is stained, the vegetative portion is counterstained with a dye of contrasting color.

b. **Reagents.**

(1) Malachite green stain. Dissolve 5 g of malachite green in a small amount of water. Add enough distilled water to make 100 ml.

(2) Safranin counterstain. Dissolve 0.5 g of safranin in a small amount of distilled water. Add sufficient distilled water to make 100 ml.

c. **Technique.**

(1) Flood the heat-fixed smear with malachite green stain and gently steam for 3-5 minutes by passing a flame under the flooded slide on a staining rack. Do not allow the stain to boil or dry.

(2) Wash the slide thoroughly with tap water.

(3) Counterstain with safranin for 30 seconds to 1 minute.

(4) Wash again and blot dry.

(5) Examine using the oil immersion objective.

d. **Interpretation.** The bacterial spores stain green, while the vegetative bacillary cells stain pink to red.

2-11. STAINING OF BLOOD SMEARS

Smears of blood and exudates or tissue impressions are sometimes prepared and examined for Borrelia recurrentis, Bacillus anthracis, or other organisms using Wright's or Giemsa's staining technique. For Wright's staining procedure, consult TM 8-227-4, Clinical Laboratory Procedures--Hematology. For Giemsa's stain, consult TM 8-227-2, Clinical Laboratory Procedures--Parasitology.

2-12. PREPARATION OF WET UNSTAINED MOUNTS FOR MICROSCOPIC EXAMINATION

Occasionally the microscopic examination of wet, unstained preparations is of value in aiding bacteriological identification.

a. **Wet Coverslip Mount.**

(1) Place a loopful of specimen or broth culture in the center of a coverslip rimmed with petroleum jelly. A faintly turbid suspension of cultural growth in a drop of saline may be substituted.

(2) Cover the droplet with a clean glass slide, gently pressing the latter down to form a thin film.

(3) Examine the preparation under reduced lighting using the high dry or oil immersion objective, whichever is required. Unstained bacteria appear as small hyaline bodies.

b. **Hanging Drop Mount.** The hanging drop mount is used to detect motility of living bacteria in pure culture. The technique for preparation and microscopic observation of hanging drop mounts is as follows:

(1) Ring a standard coverslip (20 mm square) with petroleum jelly.

(2) Place a loopful of broth culture in the center of the coverslip on the side containing the petroleum jelly.

(3) Invert a concave glass slide, placing the concavity directly over the specimen. Press to allow the petroleum jelly to form a seal. Invert.

(4) Examine the preparation microscopically under reduced lighting. Flagellar motion is evidenced- by directional movement of individual cells. Directional movement should not be confused with Brownian movement.

(5) Place all hanging drop mounts in a disinfectant solution before cleaning the slides.

c. **Darkfield Examination.** Use the wet coverslip mount technique for preparation of a wet mount of exudate material for darkfield examination. Darkfield illumination is described in MD0854, Serology.

Section II. CULTIVATION OF BACTERIA (MEDIA)

2-13. INTRODUCTION

a. In order to identify bacteria in a specimen, it is usually necessary to inoculate artificial culture media with samples of the specimen and perform studies on bacterial colonies found in the cultures after incubation. This necessitates an understanding of the growth requirements for cultivating bacteria.

b. The nutritional spectrum of bacteria varies from the self-reliant autotrophs (that is, those bacteria that are able to synthesize cellular materials from inorganic nutrients) to the more nutritionally demanding heterotrophs (which require a carbon source in the form of organic materials). It is in the latter group, the heterotrophs, that we find the parasites, both the saprophytes and the pathogens.

c. Most media are available commercially in the dehydrated form. Since dehydrated products have been economically used with considerable success in most laboratories, it is recommended that commercial media be prepared according to the manufacturer's directions.

d. For additional information about specific culture media, consult printed information provided by Difco Laboratories, Detroit, and BioQuest (Division of Becton, Dickinson and Company), Cockeysville, Maryland.

2-14. COMPOSITION OF MEDIA

a. Agar, a complex carbohydrate obtained from seaweed, is used as a solidifying agent in many media. It is inert and is not a source of nutrients. A broth (liquid) medium usually contains no agar.

b. Pep tones are nitrogenous compounds derived from specific proteins or protein mixtures by hydrolysis to provide a more available form of nitrogen for bacteria.

c. Meat extracts, meat infusions, and other natural products are sometimes used to enrich media.

d. There is a trend to the use of media that are better defined chemically. Modern media tend to consist of well-defined components, such as the peptones described in the US Pharmacopoeia and pure chemicals.

e. Culture media without glucose generally give more reliable and consistent results than media with glucose. Glucose fermentation may result in a pH that is harmful to acid-sensitive organisms. In a blood agar base medium, the presence of glucose can make it harder to differentiate between alpha and beta hemolysis. However, glucose is useful in several specific media.

2-15. HYDROGEN ION CONCENTRATION (pH)

The growth of microorganisms is very markedly affected by the pH of the medium. A proper hydrogen ion concentration must be established and maintained by use of buffering systems to ensure maximum growth of a bacterial population. The pH must be adjusted to the proper value before sterilization. Generally, a narrow pH range of 6.8 to 7.4 is considered optimal for pathogenic bacteria since most bacteria, especially pathogenic forms, grow best near the neutral point.

Section III. ENVIRONMENTAL FACTORS

2-16. TEMPERATURE

The correct temperature is very important for the proper growth of bacteria since bacteria vary considerably with respect to temperature requirements. Some bacteria grow best in a temperature range of 10^{O} to 20^{O} C; these bacteria are called psychrophilic bacteria. Other bacteria have an optimum temperature in the 30^{O} to 40^{O} C range. They are referred to as mesophilic bacteria. The thermophilic bacteria have an optimum temperature range from 50^{O} to 60^{O} C. The majority of human pathogens are mesophilic, growing best at 37^{O} C, and for this reason a constant temperature incubator adjusted to 37^{O} C (normal body temperature) and containing sufficient moisture satisfies the temperature requirement in a clinical bacteriology section. However, some authorities suggest that most incubators should be set at 35^{O} C, so that a temperature higher than 37^{O} C will be unlikely at any time.

2-17. OXYGEN REQUIREMENTS

It is essential that the proper gaseous environment be furnished when attempting to cultivate bacteria. The specific needs apply to oxygen and carbon dioxide. Often, the failure to isolate pathogenic microorganisms from clinical materials is due to inadequate provisions with respect to aeration. Bacteria are divisible into two broad groups on the basis of their oxygen requirements: (1) aerobic forms which require free oxygen for growth, and (2) anaerobic forms which require the absence of free oxygen for growth. Aerobic and anaerobic bacteria are further divided into several categories (figure 2-1).

a. **Obligate Aerobes.** An obligate aerobe must have free atmospheric oxygen in order to grow. These forms grow best on agar media in a normal atmosphere. They will also grow in the upper portion of a broth medium. Some members of the genus Bacillus are strict aerobes.

b. **Microaerophiles.** For optimal growth these forms require a greatly reduced atmosphere of oxygen such as that supplied in the lower portion of a broth medium. These organisms will also grow well on an agar plate, when incubated in an atmosphere of increased carbon dioxide (candle jar). Haemophilus species and many streptococci are microaerophiles.

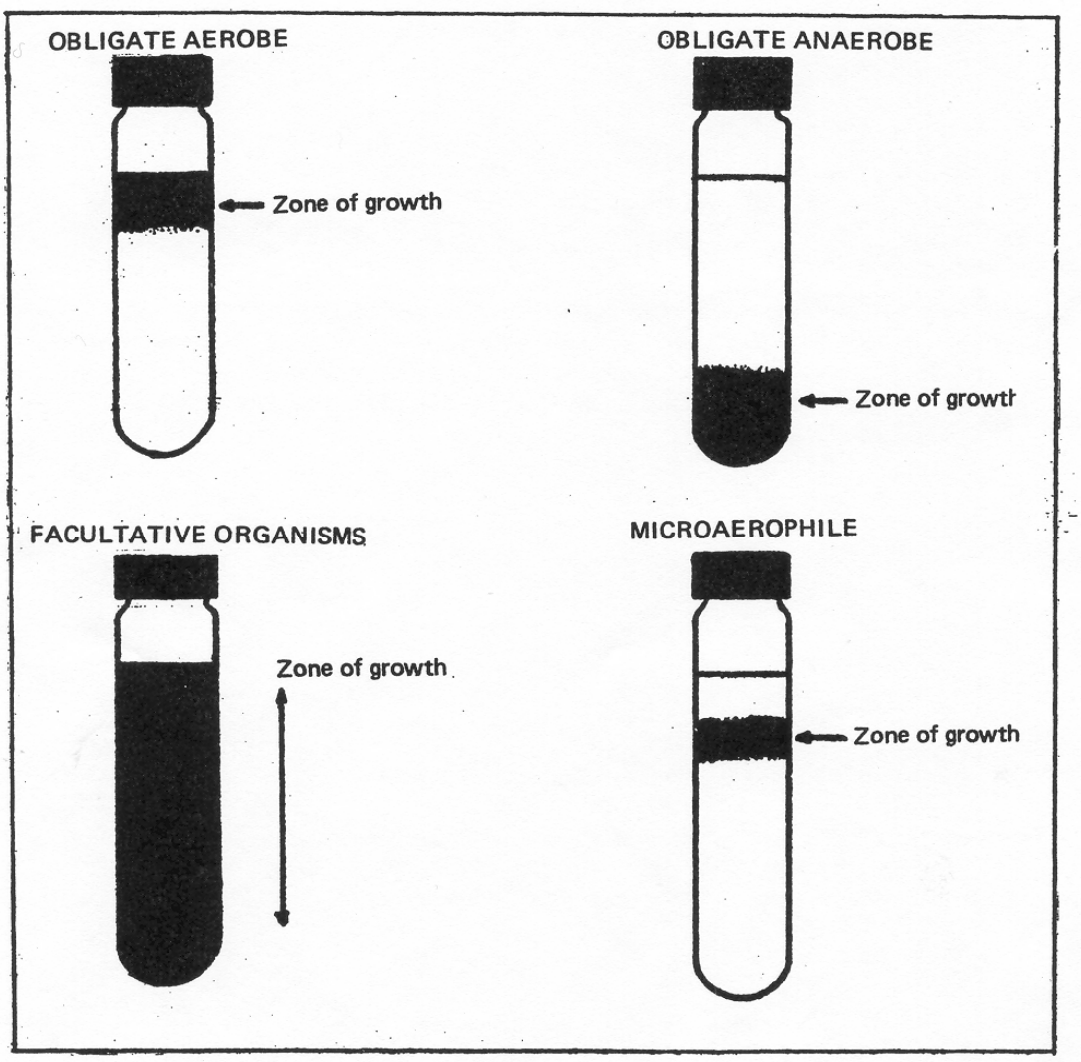


Figure 2-1. Growth in thioglycollate medium.

c. **Facultative Organisms.** Those organisms capable of adapting to either presence or absence of atmospheric oxygen fall into this group.

d. **Obligate Anaerobes.** These forms require the strict absence of atmospheric oxygen for growth. Free oxygen is toxic to obligate anaerobes because of resultant enzyme destruction or inactivation. Special measures must be taken to remove and exclude oxygen from these cultures during incubation. Some pathogenic bacteria are obligate anaerobes on primary isolation yet will adapt to aerobic conditions upon subculture. Examples of true obligate anaerobes are represented in the qenus Clostridium and some streptococci.

2-18. CARBON DIOXIDE REQUIREMENTS

Many microorganisms, aerobic and anaerobic, require a carbon dioxide concentration above normal atmospheric levels. The Gas Pak systems, discussed in the next section, utilize a Hydrogen # Carbon Dioxide Generator Envelope for anaerobes and a Carbon Dioxide Generator Envelope for aerobes. These disposable envelopes are activated by pipetting 10 ml of water into the envelope. Examples of aerobes requiring increased CO_2 are Neisseria, Brucella, and Mycobacterium.

Section IV. ANAEROBIC METHODS

NOTE: This section is adapted from V.R. Dowell, Jr., and T.M. Hawkins, Laboratory Methods in Anaerobic Bacteriology, CDC Laboratory Manual, 1974, DHEW Publication No. (COC) 74-8272.

2-19. BASIC PRINCIPLES

The anaerobic bacteria can be isolated and studied quite readily provided certain cardinal principles of anaerobic bacteriology are rigidly applied. Four of the most important considerations in the cultivation of anaerobic bacteria are:

a. Proper collection and transport of the material to be examined.

b. Culture of the material as soon as possible after collection.

c. Use of freshly prepared and properly reduced media.

d. Proper anaerobic conditions.

2-20. COLLECTION AND TRANSPORT OF CLINICAL MATERIALS

a. Proper collection and transport of clinical specimens is of primary importance in the recovery of anaerobes. The sample should be collected from the active site of infection and precautions should be taken to exclude surface contaminants and aeration of the sample. Whenever possible, tissue samples or fluid aspirates should be collected rather than swab samples. The material on swabs should never be allowed to dry out. Specimens should be placed under anaerobic conditions immediately after collection for transport to the laboratory since some anaerobes are quite oxygen-sensitive and will die rapidly in M1 aerobic environment.

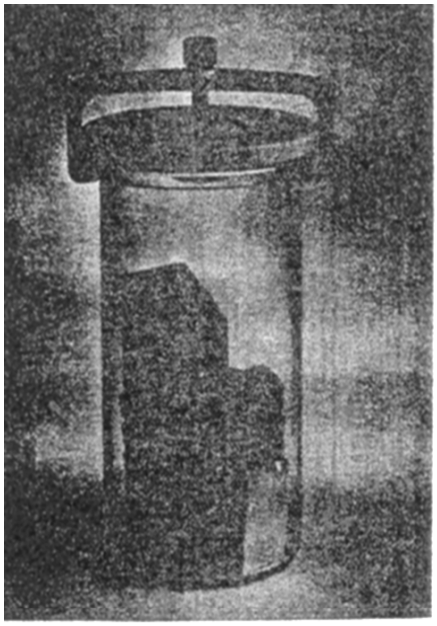
b. Sterile, rubber-stoppered transport vials and tubes containing an oxygen-free CO_2 atmosphere are available commercially. Specimens aspirated with a needle and syringe can be injected directly into the transport bottles; care must be taken to exclude any air. If necessary, a specimen tube can be opened in an upright position, the specimen or swab added, and the tube closed for transport to the laboratory. Since CO_2 is heavier than air, the CO_2 atmosphere is maintained in the transport tube. As a very minimum procedure, the material can be placed in a medium containing a reducing agent such as cysteine, or thioglycollate at room temperature for a period not exceeding 2 hours. Samples should not be refrigerated since chilling is detrimental to some anaerobes, and oxygen absorption is greater at lower temperatures.

c. All clinical material except specimens likely to be contaminated with normal flora should be routinely cultured for anaerobes. Specimens that should not be cultured include nasal swabs, throat swabs, sputum, gastric contents, skin, feces, voided or catheterized urine, and vaginal swabs.

d. For isolation of anaerobes from blood specimens, 5-10 ml of blood should be inoculated into 50-100 ml of liquid media (10 percent v/v) and the blood cultures incubated up to 21 days. Broth media containing 0.025 percent sodium polyanethol sulfonate (Liquoid) and an anaerobic or partial CO_2 atmosphere are commercially available. Tryptic soy broth, Trypticase soy broth, thioglycollate medium, and pre-reduced brain heart infusion broth designed for anaerobic blood culture all appear to be equally satisfactory. The anticoagulant liquoid may prevent the growth of some anaerobic cocci and slow the growth of some strains of Bacteroides melaninogenicus. Blood cultures should be subcultured to plating media whenever there is any obvious growth and blind subcultures made at least after 48 hours incubation and at the end of 14 days. In addition to plating on blood agar plates, it has been shown that subculturing to a selective plating medium will allow detection of anaerobes fixed with aerobic organisms in bacteremic infections.

e. Ideally, specimens should be cultured as soon as possible after collection and every effort should be made to prevent exposure of culture media to molecular oxygen. Plating medium for primary isolation should be prepared on the day it is used, or freshly prepared medium should be placed under anaerobic conditions for a period no longer than 2 weeks. Plating media can be stored in an anaerobe jar, glove box, or in an airtight cabinet containing an oxygen free CO_2 atmosphere. Liquid media containing reducing agents should be stored in the dark at room temperature in tightly capped tubes for not longer than 2 weeks.

f. Provided the media are fresh and properly reduced, successful cultivation of anaerobes can be obtained by use of the GasPak anaerobe jar (figure 2-2) or by use of an anaerobe jar with a gas replacement method (figure 2-3). At the Center for Disease Control (CDC), an anaerobe glove box system is used routinely for primary isolation of the anaerobes from clinical specimens and for subculture of colony isolates. Two excellent methods for the cultivation of anaerobes are the glove box system and the roll-streak tube system in which pre-reduced anaerobically sterilized (PRAS) media are used.



GasPak 100 (available vented and unvented) (uses 100X15-mm dishes)

GasPak 100 disposable

GasPak 150 (available vented and unvented) (uses 100X15-mm dishes)

GasPak 150 (twelve 150x15-mm dishes)

Figure 2-2. GasPak anaerobic systems (courtesy of BioQuest, Division of Becton, Dickinson and Company).

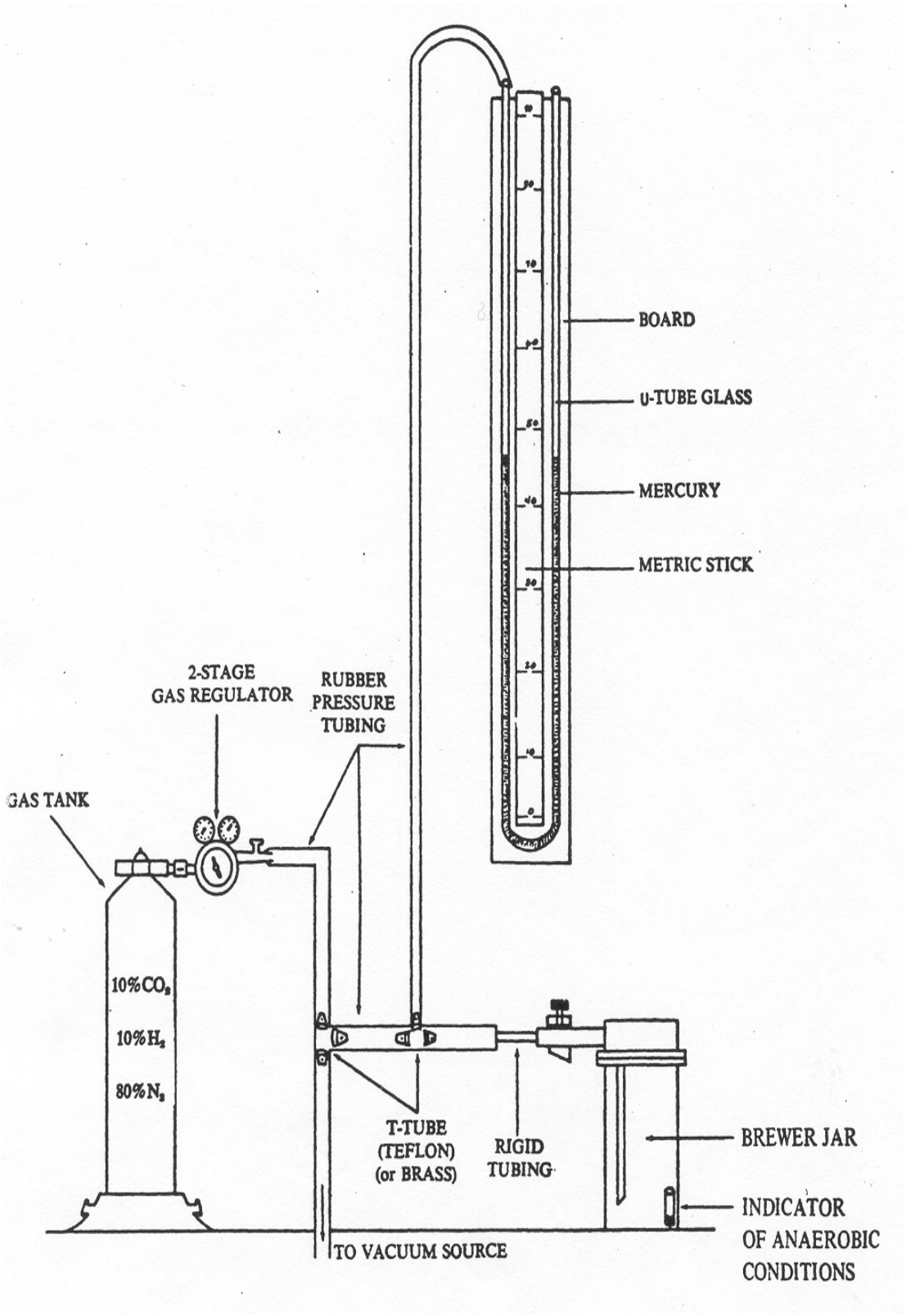


Figure 2-3. Gas replacement method with Brewer jar.

g. All specimens except blood should be gram-stained and cultured by both direct plating and enrichment procedures. All liquid or semi- solid media stored in an aerobic environment should be prereduced by heating the media for 10 minutes in a boiling water bath and cooling before inoculation. Since most clinical laboratories are not set up for the glove box or roll-streak tube systems, the following procedures are designed for use with the anaerobe culture jar.

2-21. EXAMINATION OF SMEARS

In order to gain some insight into the quantity and type of organisms in the specimen, examine a gram-stained smear. Examination of wet mounts of unstained material, acid-fast stained smears, and Giemsa-stained smears may also be helpful. Use capillary pipets to prepare smears from liquid specimens or use swabs directly. Observe and record:

a. The gram reaction, size, shape and relative numbers of organisms present.

b. The presence of spores and their shape and position in the cell.

c. Any distinctive morphological features such as branching, pseudo-branching, chaining, filaments, spherical bodies, or minute granular forms.

2-22. INOCULATION OF PRIMARY ISOLATION MEDIA

a. Inoculate primary isolation media as soon as possible after specimens are received.

(1) Fluid specimens. Use a capillary pipet to inoculate liquid or semisolid media near the bottom with one to two drops of inoculum. Place one drop on each plating medium and streak for isolation.

(2) Tissue or other solid specimens. Mince with sterile scissors, add sufficient pre-reduced broth to emulsify the specimen, add sterile sand as necessary, and grind with a mortar and pestle. Treat as a liquid specimen.

(3) Swabs. Place swab directly into liquid media and use separate swab to streak plates. If necessary, an inoculating suspension can be prepared by gently scrubbing the inoculum off a swab in approximately 2 ml of prereduced broth.

b. Heat all liquid and semisolid media in a boiling water bath for 10 minutes and cool before inoculation.

c. Inoculate one tube each of thioglycollate and chopped meat- dextrose medium (for enrichment cultures) and two blood agar plates (BAP). Add 0.5 ml of sterile rabbit serum to the thioglycollate medium after the medium has been heated and cooled. Laked blood agar plates are recommended for the isolation of Bacteroides melaninogenicus.

(1) If clostridia are suspected inoculate one egg yolk agar plate (EYA) or a plate of stiff blood agar (4 percent to 6 percent agar).

(2) If mixed bacterial populations are suspected, inoculate selective plating media, such as phenylethyl alcohol blood agar and/or kanamycin-vancomycin-menadione blood agar. Promomycin or neomycin may be substituted for kanamycin.

2-23. INCUBATION AND SUBCULTURE

a. Incubate cultures at 35º to 37º C as follows.

(1) Incubate chopped meat and thioglycollate media in an anaerobe jar for 24-48 hours. Loosen caps to allow exchange of gasses.

(2) Incubate one BAP in a candle jar to determine aerobic flora.

(3) Incubate one BAP, EYA plate, and selective plating media in an anaerobe jar for a minimum of 48 hours, preferably 3 to 5 days. To absorb excess moisture in plates, use glass Petri dishes (100 x15 mm) with metal covers containing absorbent discs or:

(a) Add 2-3 drops of glycerin to the lid of each plate.

(b) Place a piece of filter paper in the lid.

(c) Replace bottom of plate, pressing the filter paper into the dish top.

b. After 24 hours incubation, examine enrichment cultures. If no growth is apparent, re-incubate cultures.

(1) Prepare and examine gram-stained preparations from thioglycollate and chopped meat-dextrose cultures.

(2) Remove inoculum from near the bottom of the culture tubes with a capillary pipet and inoculate plating media for subculture.

(a) Inoculate two blood agar plates from either the thioglycollate or chopped meat-dextrose cultures.

1 If clostridia are suspected~ inoculate an egg yolk agar plate or stiff blood agar.

2 If the culture contains a mixture of organisms, inoculate selective plating media.

(b) Incubate plates in a candle jar or anaerobically as outlined in the preceding paragraph.

(3) Re-incubate enrichment cultures if organisms seen on direct smear are not present in cultures.

2-24. PROCEDURE AFTER INCUBATION

a. Examine anaerobic and CO_2 plates with a hand lens and dissecting microscope.

(1) Observe and record the action on blood and egg yolk, the size and shape of colonies (figure 2-4).

(a) Prepare gram-stained smears for comparison of colonies on the different plates. Record shape and location of any spores observed.

(b) Colonies on egg yolk agar may be used to test for catalase by adding a drop of 3 percent H_2O_2 to a suspension of organisms on a slide. Expose the EYA plates to air for at least 30 minutes before testing for catalase. Do not use colonies from blood agar plates to test for catalase.

(2) Determine the number of different colony types on the anaerobe plates.

(a) For each colony to be transferred, pre-reduce one tube of chopped meat-dextrose medium and one tube of thioglycollate medium by heating the media in a boiling water bath for 10 minutes. Cool before use.

(b) Using a needle with a small loop or a heat sealed 9-inch Pasteur capillary pipet, fish each different colony and inoculate a tube of chopped meat-dextrose medium and a tube of thioglycollate medium. (Chopped meat medium is best for culturing the clostridia, and the enriched thioglycollate medium is more suitable for the nonspore-forming anaerobes.) If anaerobes other than clostridia are suspected, add 0.5 ml of sterile rabbit serum to the thioglycollate medium.

(3) Incubate chopped meat and thioglycollate media in an anaerobe jar for 24-28 hours.

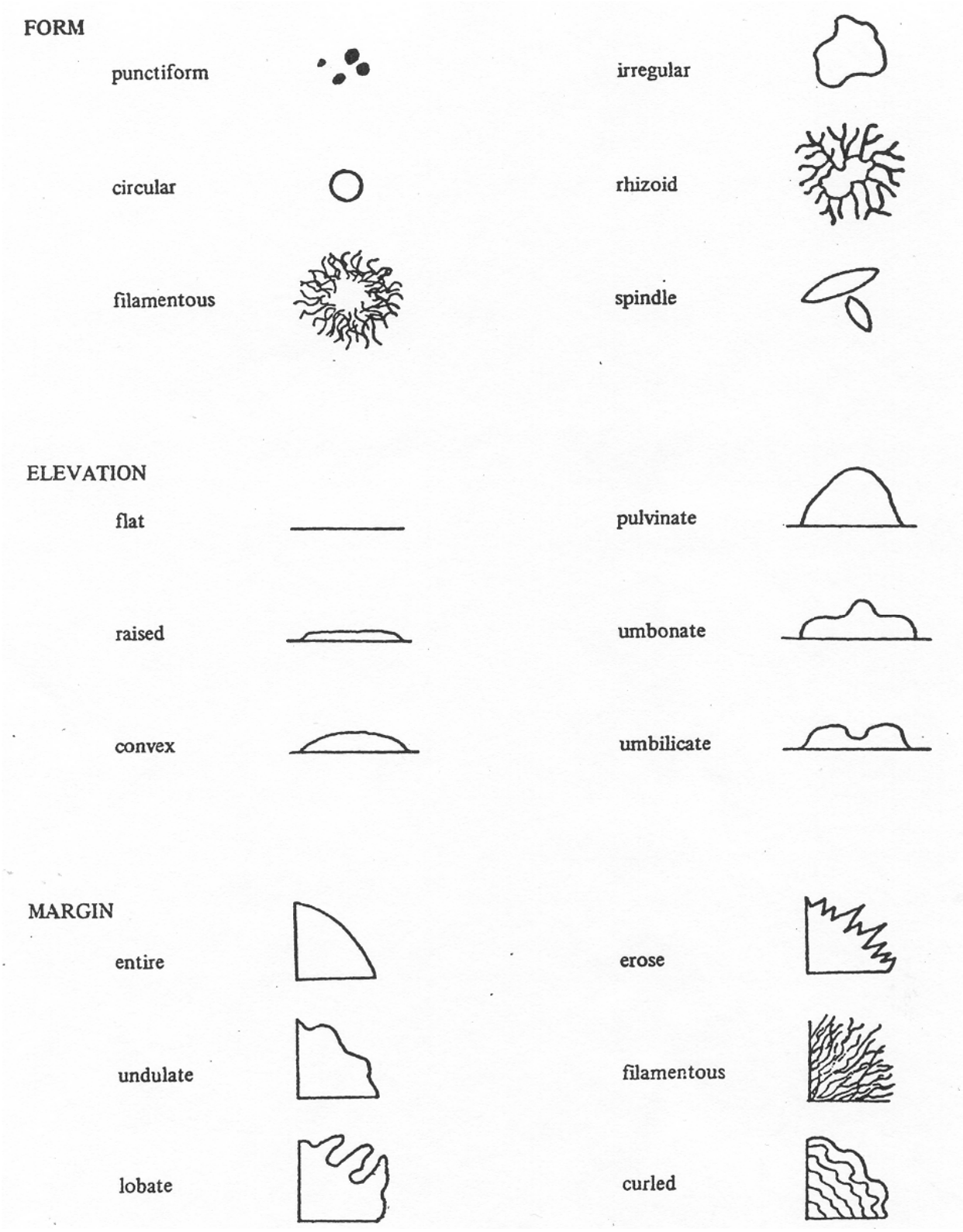


Figure 2-4. Colony characteristics.

b. Be sure to have at least one representative colony of each morphological type seen on the original smear. If necessary, restreak plating media to obtain isolated colonies.

c. Examine plates inoculated with enrichment cultures after incubation. Subculture any colony types not isolated from direct plates to prereduced chopped meat-dextrose and thioglycollate media.

d. Examine thioglycollate and chopped meat subcultures from isolated colonies. If pure, use these cultures to inoculate appropriate differential media for identification of the isolates.

Section V. ANTIBACTERIAL AGENTS, STERILIZATION, AND ASEPTIC TECHNIQUE

2-25. ANTIBACTERIAL AGENTS

Sterility may be defined as freedom from all living organisms, and sterilization is the process of killing or removing microorganisms from the environment. An agent exerts a bactericidal effect if it kills bacteria, while a bacteriostatic agent inhibits bacterial reproduction. On removal of the bacteriostatic agent, bacterial reproduction resumes. A disinfectant is a bactericidal agent that acts as a general protoplasmic poison. Because disinfectants are generally very toxic, their use is restricted to fomites (nonliving objects such as glassware, bench tops, and floors). An antiseptic is a bactericidal agent that is less potent than a disinfectant. Antiseptics are used topically, externally, on the skin. An understanding of these terms is necessary for consideration of the effects of physical and chemical agents upon bacteria.

a. **pHisoHex.** pHisoHex is the brand name of a detergent that is used as an antiseptic. This detergent contains the phenolic derivative, hexachlorophene, which acts as a bactericidal agent. Small amounts of this detergent should be used to wash the hands whenever bacterial cultures or clinical specimens have been handled.

b. **Wescodyne.** Wescodyne is the brand name given to a solution of active iodine. It acts as an oxidizing agent and is used as a general disinfectant. It is further characterized as nonirritating to the skin.

c. **Phenol.** Phenol is an organic compound whose germicidal activity is taken as the standard for all disinfectants; that is, the activity of other disinfectants is compared to that of phenol. A 5 percent solution of phenol readily destroys spores and vegetative cells. It is used on inanimate or nonliving objects such as glassware, bench tops, and floors.

d. **Alcohol.** Alcohol is used extensively as an antiseptic. It is relatively ineffective as a bacterial agent, but exerts its maximum effect at a concentration of 70 percent.

2-26. GENERAL COMMENTS ON STERILIZATION

a. Bacteriological identification requires that pure cultures of microorganisms be studied. Since microorganisms are ubiquitous, all materials used in the cultivation of bacteria must be subject to preliminary sterilization.

b. The fundamental principle of media sterilization is to remove or destroy all living material on or within a medium without rendering it ineffective for cultivating the desired microorganisms. The methods most commonly used involve moist or dry heat and filtration. The particular sterilization method employed is governed by the items to be sterilized and their intended use.

c. The usual items of glassware must be scrupulously clean. Flasks and tubes are plugged with nonabsorbent cotton to prevent entry of bacteria after sterilization. Small portions of cotton are inserted in the mouthpiece of pipets that are subsequently wrapped in paper or placed in metal canisters. Petri dishes must be wrapped in paper or placed in canisters. All materials are wrapped to preclude contamination after sterilization. It is a good practice to cap cotton plugs of flasks with paper or foil. Glassware prepared in this manner will remain sterile up to 30 days if properly stored.

2-27. MEDIA PREPARATION

Just as it is important to observe aseptic technique in the processing and culturing of specimens, it is equally important to prepare the media used in bacteriology with great care. The results obtained depend directly on the quality of the media used. Before autoclaving, the flask containing the medium should be covered. This cover is kept in place except when the medium is actually being poured after autoclaving. Before the medium is poured from the flask, and occasionally while pouring, the mouth of the flask should be flamed to kill any air contaminants that may have landed on the mouth. Plates are poured by lifting the lip of the Petri plate only enough to pour in the agar. The flask must not be allowed to touch the plate while pouring, and as soon as a plate has been poured, the lid must be put back on. Liquid media are prepared either by pouring or dispensing into tubes and then autoclaving, or by pipetting sterile media into sterile tubes.

2-28. HEAT

In any discussion of the effect of heat upon bacteria, it must be realized that a time-temperature relationship exists in all cases. In this regard, if vegetative bacterial cells are exposed to a temperature of 55^{O} to 58^{O} C for 30 minutes, all psychrophiles and mesophiles will be destroyed, while some thermophiles will survive. The process of pasteurization is based upon this time-temperature relationship. Heat may be applied in a dry or wet form.

a. **Dry Heat.** The use of dry heat to control bacteria is frequently employed in the bacteriology laboratory. The flame of the Bunsen burner is used to sterilize bacteriological loops and needles. Contaminated materials that will burn are destroyed by burning. The hot air oven is used to dry glassware and to sterilize glassware and metalware. Sterilization is brought about by employing a temperature of 170º to 180º C for 1 1/2 to 2 hours. Heat labile substances such as culture media, paper, rubber, plastic items, and non-heat-resistant glassware cannot be sterilized by using the hot-air oven.

b. **Moist Heat.** Control systems based upon the use of moist heat are used extensively. Moist heat rather than dry heat is used in the sterilization of culture media.

(1) Boiling water. The most basis system using moist heat to control bacteria is seen in the practice of placing surgical instruments in boiling water. This procedure does not always provide sterile conditions. Ideally, boiling water provides a temperature of 100º C and although vegetative bacterial cells are destroyed by such treatment, spores of bacteria are resistant to boiling water. Accordingly, boiling is not a recommended procedure, and it is not used in the bacteriology laboratory to establish sterile conditions.

(2) Free-flowing steam. Free-flowing steam finds limited use in the bacteriology laboratory as a means of sterilizing media. It must be remembered that in terms of temperature, steam is formed at 100^{O} C. Any sterilization procedure based upon the utilization of free-flowing steam will be limited to the destruction of vegetative cells. For this reason, media to be sterilized by tyndallization, the name given to the procedure of sterilization by the use of free-flowing steam, must be treated on 3 successive days. Most spores present in the media would germinate, and the resultant vegetative cells would be susceptible to destructive action of free-flowing steam. The Arnold sterilizer is based upon the free-flowing steam principle.

(3) Steam under pressure. At this point it should be apparent that if spores are to be destroyed along with vegetative cells, a temperature higher than 100º C is required. Temperatures higher than 100º C are made possible by placing steam under pressure; the autoclave is the name of the instrument whose operation is based upon steam under pressure. It is the increased temperature that destroys microorganisms; the pressure acts to increase the temperature of the steam. Usually the items to be sterilized are exposed to a temperature of 121º C for 15 minutes at a setting of 15 pounds pressure. (Exposure time starts after the desired temperature level and pressure are reached.) It must be remembered, however, that the increased temperature of the autoclave may result in the breakdown of thermolabile substances such .as urea and carbohydrates. When media containing these substances are; to be sterilized, adjustments in the sterilization procedure are required. Autoclaving is the most extensively used method for sterilizing culture media.

2-29. FILTRATION

Sterilization by filtration represents a mechanical means of removing bacteria from liquids. When the relative size of bacteria and spores is recalled, it should be apparent that the porosity of the filter that is used must be extremely small. The Seitz filter and the membrane filter are examples of very fine filters that are used in the bacteriology laboratory. The use of these filters is recommended for the sterilization of liquids containing thermolabile substances such as carbohydrates, urea, and sera.

2-30. RADIATION

Bacteria, like all living systems, are susceptible to the lethal effects of radiation. In the laboratory, ultraviolet (UV) light sources are sometimes built into isolation hoods. Special UV lighting devices are sometimes installed in rooms where highly infectious specimens, such as those from tuberculosis patients, are handled. Exposure of specimens or equipment must be direct and sufficiently prolonged. (Severe damage to the eyes can result from even a short exposure to ultraviolet rays. Highly penetrating rays such as x-rays and gamma rays are not routinely used in the medical bacteriology laboratory.)

2-31. GENERAL COMMENTS ON ASEPTIC TECHNIQUE

In the bacteriology section of a laboratory, the specialist is constantly exposed to microorganisms that can and do cause disease in man. Aseptic technique is a manipulative skill that prevents self-infection when working with pathogenic microorganisms and also prevents the introduction of extraneous microorganisms into a system. This skill is applied when dealing with bacteria or with anything that can come into contact with bacteria. The purpose of aseptic technique is to protect the laboratory specialist and his co-workers, and to prevent contamination of the specimen with which he is working.

2-32. WORKING AREA

a. The working area must be decontaminated before starting the day's work and again at the completion of the day. In addition, if a specimen should be spilled onto the working area, the affected area should be immediately decontaminated. One way to accomplish decontamination of working space is to soak a paper towel with the decontaminant and then wipe the working area with this solution and allow to air dry. Ultraviolet light can also be effectively used, but its use is ordinarily restricted to a specific area, such as an inoculating hood, or to nighttime use.

b. Unauthorized personnel should be forbidden access to the bacteriological section. Doors should remain closed.

2-33. DISPOSAL OF CONTAMINATED MATERIALS

The media, specimens, and equipment that are used in the processing of specimens must be correctly disposed *of* to avoid the possibility of infecting yourself or your co-workers, and to avoid the possibility of contamination of other specimens. This material may be safely decontaminated *by* incineration, autoclaving, or by immersion in disinfectant solutions.

a. Loops and needles are sterilized by incineration. Swabs and disposable equipment may also be incinerated, but should not be incinerated by a Bunsen burner.

b. Autoclaving can be used on all contaminated materials such as swabs, Petri dishes, and test tubes. Swabs and other disposable materials should then be discarded. Glass Petri dishes, tubes, and closures should be cleaned and sterilized for reuse.

c. After use, glassware is placed in a disinfectant solution. Next, it is autoclaved, washed, plugged with cotton (as applicable), and sterilized in pipet canisters or individual packets for reuse.

d. The method used by any laboratory depends on the procedures of that laboratory, but strict attention must be paid to aseptic technique, regardless of the procedure used.

2-34. PERSONAL CLEANLINESS

A laboratory specialist working in the bacteriology section of a clinical laboratory must take measures to protect himself. To adequately accomplish this, it is frequently necessary to wash your hands in disinfectant solution or with surgical soap. It is always necessary to practice aseptic technique. You should never put objects such as fingers or pencils into your mouth. Avoid mouth pipetting. If you should accidentally spill a specimen on yourself, you must decontaminate yourself with a noncaustic disinfectant solution such as Wescodyne. To ignore any of these precautions is to invite trouble in the form of an infection or illness.

Section VI. ISOLATION OF BACTERIA

2-35. INTRODUCTION

a. Disease-producing bacteria usually occur in specimens in association with other bacteria, rather than in the pure state. In laboratory identification of microorganisms it is necessary that pure cultures (cultures containing only a single species) be studied.

b. To secure a pure culture of a given organism from a specimen or sample containing mixed flora, it is necessary to isolate a single cell from all other cells present. The cell is cultivated in such a manner that its collective progeny remain isolated.

c. Two common methods used to inoculate specimens or broth cultures to agar media are the streak plate method and the pour plate method.

2-36. INOCULATING LOOPS AND NEEDLES

The tools used in these techniques are the loop and the needle.

a. The inoculating loop and the inoculating needle are composed of platinum or nichrome wire affixed to a handle. The needle is used to transfer colonies from one broth medium to another or to an agar medium.

b. During use, the loop or needle must never be allowed to touch the outside or lip of a container since contamination may result. Contamination gives a distorted picture of the contents of the specimen that is being cultured. When using a needle to pick a colony from an agar medium, touch the needle to the top center of the colony, avoid visible contaminants, and do not dig into the agar.

c. Before a loop or needle is used, it must be sterilized. It is held downward at a 45º angle within the blue part of the flame of a Bunsen burner and is heated to red hot, starting at the base of the wire next to the handle and moving slowly toward the tip. If the loop or needle is not clean and dry, it should be slowly heated at first to avoid spattering and contamination of the surroundings. After being, sterilized, the loop or needle is cooled for approximately 1 to 20 seconds to prevent heat destruction of the microorganisms in the specimen.

d. When not in use, the loop or needle is kept in a rack or a special stand. It should never be placed on a working surface.

2-37. STREAK PLATE

a. The plate is kept in an inverted position during processing and incubation. Only the part of the Petri dish containing the medium is picked up when working with the dish in order to prevent contamination of the surface of the plating medium and therefore contamination of the specimen. This is done whether the streaking is for isolation or for sensitivity studies. The Petri dish should be labeled with the specimen number or patient name, (the date and time of inoculation, and the initials of the laboratory specialist doing the inoculating). This is done prior to inoculation to prevent any possibility of the interchange of plates and the reporting of incorrect results.

b. A loopful of inoculum is collected on a flame-sterilized wire loop and streaked over approximately one-quarter of the agar surface. After flaming the loop again and without collecting more inoculum, the plate is rotated slightly and another quadrant of the agar surface is streaked, overlapping the original quadrant as shown in figure 2-5. This process of diluting and spreading the inoculum over the medium is continued until the entire agar surface is covered. As the streaking continues, fewer and fewer cells

remain on the loop, and finally single cells are deposited on the agar. Each isolated cell will give rise to a visible colony under suitable environmental conditions. If clinical materials on cotton swabs are to be cultured, the swab is rolled over a small area of the agar surface at the edge of the plate. With a wire loop the inoculum is spread over the four quadrants of the agar surface in the previously described manner (figure 2-5).

c. The streak plate method is the must common means of securing isolated colonies, and when properly done is just as reliable as and much more rapid than the pour-plate method. Although any agar base medium may be used, blood agar streak plates are usually employed for primary isolation of pathogenic bacteria. After inoculation, the plates are ordinarily incubated at 37º C for 18-24 hours in the inverted position. Growth of isolated colonies is examined grossly and microscopically for characteristics of various genera and species. Using a wire needle, pure cultures are obtained by picking growth from the center of the colony and subculturing to suitable broth or agar media.

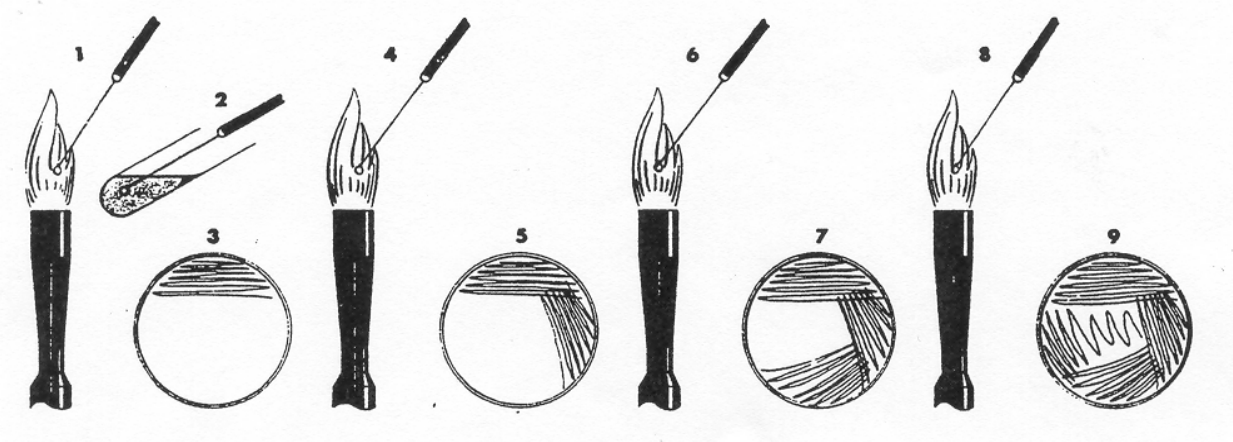


Figure 2-5. Streak plate method.

2-38. POUR PLATE METHOD

a. At times it may be necessary to utilize a pour plate technique for securing isolated colonies. To accomplish this, culture tubes containing approximately 12 ml of sterile infusion agar or other suitable medium, are placed in boiling water to melt the agar. After cooling the medium to about 48^{O} C in a water bath, approximately 0.7 to 1.0 ml of sterile, defibrinated blood is aseptically added to each tube of melted agar. Each blood medium mixture is then inoculated with a sample of the appropriately diluted specimen, mixed well by twirling the tube, and dispensed to sterile Petri dishes. It is important that the inoculum be diluted properly in preparing pour plates when many organisms are observed in the gram smears of the specimen. This dilution may be accomplished by inoculating a loopful of the original specimen to 5 or 6 ml of sterile broth or normal saline. After mixing thoroughly, one loopful of this dilution is inoculated to the melted blood agar.

b. Upon media solidification, individual cells in the inoculum are immobilized in various areas of the agar. During incubation each cell will multiply to form a visible colony. Blood agar pour plates are primarily used for determining the type of hemolysis produced by strains of streptococci. When subcultures or stains are to be prepared from pour plates cultures, it is necessary to secure growth from individual colonies within the agar using a sterile needle.

2-39. TEST TUBE CULTURES

After isolated colonies are obtained by the streak plate or pour plate technique, it is often necessary to subculture growth to tubed media to permit further study. Tubed media are prepared by dispensing broth or agar media into appropriate test tubes. The tubes are plugged with small sections of rolled cotton that are bent in the middle and inserted in the tube 2.5 cm inside the tube and should project at least 2 cm outside the lip of the tube. Cotton plugs should fit snuggly, but not tight that difficulty will be experienced in removing and replacing the plug during bacteriological manipulations. After sterilization, broth and agar stab tubes are allowed to cool in the upright position. Agar slants are prepared by inclining the tubes of melted agar medium on a tabletop until solidification occurs. To prepare pure cultures, each type of medium is inoculated as follows:

a. **Liquid Cultures (Broth).** Using a wire loop, emulsify a small amount of growth on the moist wall of the tube just above the liquid and wash down by tilting the tube. If the inoculum is liquid, a loopful is simply placed in the broth and dispersed by gentle agitation.

b. **Slant Cultures.** Slant cultures are prepared by streaking the inoculum over the slant surface from bottom to top. If the slant contains some water of condensation at its base, do not spread the water over the surface of the slant since the resulting growth will not yield characteristic colony appearance.

c. **Stab Cultures.** Stab cultures are made with a straight needle into tubes of unslanted solid or semisolid medium. The stab line, centered without lateral movement, should extend approximately one-half to two-thirds the depth of the medium.

Continue with Exercises

EXERCISES, LESSON 2.

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. A swab specimen should be used first to make a smear and then to inoculate a culture.

   a. True.

   b. False.

2. When bacterial smears are to be prepared from growth on solid medium, a small amount should be emulsified in a loopful of water on the slide.

   a. True.

   b. False.

3. Acid-fast bacilli possess large amounts of _____ within their cells.

   a. Starches.

   b. Proteins.

   c. Lipids.

   d. Water.

4. The procedure to follow in the Ziehl-Neelsen acid fast stain is to:

    a. Stain with steaming carbolfuchsin, counterstain with methylene blue, decolorize with acid-alcohol.

    b. Stain with steaming carbolfuchsin, decolorize with acetone-alcohol, counterstain with methylene blue.

    c. Stain with steaming carbolfuchsin, decolorize acid-alcohol, counterstain with methylene blue.

    d. Stain with methylene blue, decolorize with acid-alcohol, counterstain with teaming carbolfuchsin.

5. Using the Ziehl-Neelsen acid-fast stain, overheating results in:

    a. A more intense color.

    b. Acid-fastness *of* al-l organisms.

    c. The need to use much more acid-alcohol.

    d. A diminished color.

6. What is used instead of heat in Kinyoun's modification of the Ziehl-Neelsen acid-fast stain to achieve penetration of bacteria with the primary stain?

    a. A decolorizer.

    b. Wetting agent.

    c. A counter stain.

    d. Acid-alcohol.

7. All of the following reagents are used in the capsule stain (Hiss method) EXCEPT:

    a. India ink.

    b. Normal serum.

    c. 1% crystal violet.

    d. 20% copper sulfate.

8. For the flagella stain, a smear is air-dried but not heat-fixed.

    a. True.

    b. False.

9. The most widely used staining procedure in the bacteriological laboratory is the:

    a. Gram stain.

    b. Acid-fast stain.

    c. Methylene blue stain.

    d. Capsule stain.

10. What color are gram-positive cells after completion of the gram stain?

    a. Red.

    b. Black.

    c. Green.

    d. Purple or blue.

11. Methylene blue stain is used to selectively stain:

    a. Capsules.

    b. Flagella.

    c. Metachromatic granules.

    d. Nucleoids.

12. Upon completion of the Wirtz-Conklin spore stain, spores are colored _____ and bacilli are colored _____.

    a. Pink or red; green.

    b. Green; pink or red.

    c. Red; blue or purple.

    d. Blue or purple; red.

13. In the selection of media for the cultivation of bacteria, there is a trend toward the use of media which:

    a. Are better defined chemically.

    b. Contain gelatin rather than agar.

    c. Contain more meat extracts and other natural products.

    d. Are easier to prepare directly from natural products.

14. Most pathogenic bacteria grow best at a pH near:

    a. 3.0.

    b. 4.5.

    c. 7.0.

    d. 9.0.

15. Bacteria which grow best at body temperature are termed:

    a. Aerobic.

    b. Mesophilic.

    c. Thermophilic.

    d. Psychrophilic.

16. Most human pathogenic bacteria are:

    a. Psychrophilic.

    b. Mesophilic.

    c. Thermophilic.

17. If an organism grows only in an environment containing no free oxygen, it is:

    a. A facultative anaerobe.

    b. A facultative aerobe.

    c. An obligate anaerobe.

    d. An obligate aerobe.

18. The use of media containing cysteine or thioglycollate without complete anaerobic conditions should NOT last longer than:

    a. 2 hours.

    b. 12 hours.

    c. 24 hours.

    d. 48 hours.

19. All of the following specimens should not be routinely cultured for anaerobes EXCEPT:

    a. Feces.

    b. Blood.

    c. Sputum.

    d. Nasal and throat swabs.

20. When should anaerobic blood cultures be subcultured?

    a. All of the below.

    b. After 48 hours (blind subculture).

    c. After 14 days (blind subculture).

    d. Whenever there is evidence of growth.

21. Plating medium (for primary isolation of anaerobes) CANNOT be used on the day of its preparation. Under what condition can it be stored up to 2 weeks?

    a. Aerobic.

    b. Anaerobic.

22. A bactericidal agent is one which:

    a. Inhibits bacterial reproduction.

    b. Kills bacteria.

    c. Accelerates bacterial growth.

    d. May only be applied to the skin.

23. A bacteriostatic agent is one which:

    a. Inhibits bacterial reproduction.

    b. Kills bacteria.

    c. May only be used topically.

    d. Is a protoplasmic poison..

24. Which of the following bactericidal agents is used as the basis for standardizing germicidal activities for all disinfectants?

    a. Alcohol.

    b. pHisoHex.

    c. Phenol.

    d. Wescodyne.

25. Alcohol is a rather ineffective bactericidal agent. It exerts its maximum effect at a concentration of:

    a. 70 percent.

    b. 60 percent.

    c. 50 percent.

    d. 40 percent.

26. After sterilization, materials are packed or wrapped to preclude:

    a. Breakage.

    b. Heat damage.

    c. Chemical breakdown.

    d. Contamination.

27. Spore-forming bacteria are more resistant to heat than are most other bacteria.

    a. True.

    b. False.

28. Any sterilization procedure based on tyndallization is limited to:

    a. The destruction of vegetative bacterial cells.

    b. The destruction of spores.

    c. Use on equipment only.

    d. Use on agar type media only.

29. The temperature and pressure necessary for sterilization in the autoclave are:

    a. 100º C, atmosphere pressure.

    b. 100º C, 15 pounds pressure.

    c. 121º C, atmospheric pressure.

    d. 121º C, 15 pounds pressure.

30. Which of the following is the most extensively used method for sterilizing culture media?

    a. Dry heat.

    b. Autoclaving.

    c. Filtration.

    d. Tyndallization.

31. Thermolabile substances are sterilized by:

    a. Dry heat.

    b. Autoclaving.

    c. Filtration.

    d. Tyndallization.

32. During processing and incubation, the agar plate is kept in _____ position.

    a. An open.

    b. A slanted.

    c. A vertical.

    d. An inverted.

33. Before inoculation, an agar plate is labeled with:

    a. All of the below.

    b. Date and time.

    c. Laboratorian's initials.

    d. Specimen number or patient's name.

34. When we streak a clinical specimen on an isolation medium, we:

    a. All of the below.

    b. Flame the loop between each direction of the streaking process.

    c. Deposit progressively smaller amounts of the specimen on the agar surface.

    d. Use either a cotton swab or a flame-sterilized loop for the initial inoculation.

35. Blood agar pour plates are primarily used for determining the type of hemolysis produced by strains of:

    a. Streptococci.

    b. Staphylococci.

    c. Pneumococci.

    d. Haemophilus.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES, LESSON 2.

1. b (para 2-2a(6))
2. a (para 2-2c)
3. c (para 2-3a)
4. c (para 2-3c)
5. d (para 2-3e)
6. b (para 2-4a)
7. a (para 2-6b)
8. a (para 2-7c(4))
9. a (para 2-8)
10. d (para 2-8d(l))
11. c (para 2-9a)
12. b (para 2-10d)
13. a (para 2-14d)
14. c (para 2-15)
15. b (para 2-16)
16. b (para 2-16)
17. c (para 2-17d)
18. a (para 2-20b)
19. b (para 2-20c)
20. a (para 2-20d)

21. b (para 2-20e)

22. b (para 2-25)

23. a (para 2-25)

24. c (para 2-25c)

25. a (para 2-25d)

26. d (para 2-26c)

27. a (para 2-28b(1))

28. a (para 2-28b(2))

29. d (para 2-28b(3))

30. b (para 2-28b(3))

31. c (para 2-29)

32. d (para 2-37a)

33. a (para 2-37a)

34. a (para 2-37b)

35. a (para 2-38b)

End of Lesson 2

LESSON ASSIGNMENT

LESSON 3 Common Tests and Gram-Positive Cocci.

TEXT ASSIGMMENT Paragraphs 3-1 through 3-32.

LESSON OBJECTIVES Upon completion of this lesson, you should be able to:

3-1. Make correct paired associations among the name of the test, its principle, its diagnostic significance, possible results, and interpretation of results for each of the following:

a. Bile solubility test.
b. Carbohydrate fermentation test.
c. Catalase test.
d. Citrate test.
e. Coagulase test.
f. Ferric chloride test.
g. Gelatin liquefaction test.
h. Hydrogen sulfide test (lead acetate paper test).
i. Indole test.
j. Methyl red test.
k. Nitrate reduction test.
l. Oxidase test.
m. Urease test.
n. Voges Proskauer test.

3-2. Make correct paired associations among names of organisms and discriminating characteristics of colony morphology, gram morphology, biochemical reactions, biochemical reactions, pathogenicity, and interpretation of results for:

a. Staphylococcus aureus and staphylococcus epidermidis.
b. Hemolytic groups and Lancefield groups of streptococci.
c. Streptococcus (Pneumococcus) pneumoniae.

SUGGESTION After studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 3

COMMON TESTS AND GRAM-POSITIVE COCCI

Section I. COMMON TESTS

3-1. BILE SOLUBILITY TEST

a. **Principle.** Pneumococci (Streptococcus pneumoniae) are lysed in the presence of bile salts; other streptococci are not lysed. This test is used to distinguish between pneumococci and other alpha hemolytic streptococci.

b. **Reagents and Media.**

(1) Two percent sodium deoxycholate (bile salt). Dissolve 2 g of sodium deoxycholate in a small amount of distilled water. Add sufficient distilled water to make a total volume of 100 ml.

(2) Physiological saline. Dissolve 8.7 g of sodium chloride (NaCl) in approximately 100 ml of distilled water. Add enough distilled water to make a total volume of 1,000 ml. As required, pour into flasks, plug, and sterilize in an autoclave.

(3) Phenol red indicator.

(4) 0.1N sodium hydroxide. Using a pipet, transfer 1 ml of 10N or 40 percent sodium hydroxide to a 100-ml volumetric flask and add enough distilled water to make 100 ml.

(5) Thioglycollate broth or Todd-Hewitt broth.

c. **Technique.**

(1) Inoculate a tube of thioglycollate broth or a tube of Todd-Hewitt broth with the bacteria in question.

(2) Incubate the broth culture for 18-24 hours at 37º C.

NOTE: An alternative procedure is to prepare a heavy suspension of the bacteria to be tested in 1.0 ml of physiological saline and proceed to step 3.

(3) Because of the fermentation of glucose and the resulting lactic acid formation in streptococcal or pneumococcal cultures, adjustment of pH to neutral is necessary before proceeding with the test. Adjust the pH of the broth culture (or saline suspension) by adding one drop of phenol red indicator (or other suitable indicator) to the latter and then titrating with a few drops of 0.IN sodium hydroxide until a neutral pH is obtained. If phenol red is used, the neutral pH is manifested by a pink color.

(4) Place 0.5 ml of the neutralized suspension into each of two clean Kahn tubes.

(5) Label one tube test and add to this tube 0.5 ml of 2 percent of sodium deoxycholate.

(6) Label the second tube control and add to this tube 0.5 ml of physiological saline.

(7) Place both tubes in the incubator at 37º C and observe at 1, 2, and 3 hours for clearing in the tube labeled test (the tube containing the sodium deoxycholate).

d. **Interpretation.**

(1) If the organism is Streptococcus pneumoniae, clearing should occur in the tube containing the sodium deoxycholate within the 3-hour incubation period. Other alpha streptococci cultures remain turbid like the control.

(2) Regard as negative all tubes that do not clear within the 3 hours of incubation.

(3) The cultures must be neutralized (step (3)) because sodium deoxycholate in an acid solution may form a precipitate or gel that interferes with the reading of the test.

3-2. CARBOHYDRATE FERMENTATION TEST

a. **Principle.** In media containing carbohydrates, certain bacteria form acids. Some of these bacteria will produce gases, in addition to acids, by furthering the fermentation process.

b. **Reagent and Medium.** Use purple broth base containing 1 percent concentration of the desired carbohydrate. (This medium contains bromcresol purple as an indicator.)

c. **Technique.**

(1) Inoculate the organism to a small tube containing an inverted Durham tube in approximately 5 ml of the 1 percent carbohydrate base.

(2) Incubate the tube for 18 to 48 hours at 37º C.

(3) Observe for evidence of acid and gas production.

d. **Interpretation.** Fermentation to acid is evidenced by the medium's being converted from purple to distinct yellow. Yellow broths should be examined carefully for the possible presence of gas, which is observed as a bubble within the inverted Durham tube.

3-3. CATALASE TEST

a. **Principle.** Staphylococci produce the enzyme catalase which, when mixed with hydrogen peroxide, will liberate oxygen from the hydrogen peroxide with the occurrence *of* vigorous bubbling.

b. **Reagent.** Use 30 percent hydrogen peroxide (superoxol).

c. **Interpretation.**

(1) Immediate bubbling in the drop of hydrogen peroxide signifies a positive test.

(2) Staphylococci are catalase positive. Absence of immediate bubbling signifies a negative test. Streptococci and pneumococci are catalase negative.

(3) If the loop digs into blood agar medium, blood cells from blood agar may produce a false-positive reaction.

(4) Platinum wire may produce Nichrome wire can be used satisfactorily.

d. **Technique.**

(1) Place a drop of hydrogen peroxide on a clean glass slide.

(2) Pick the colony (colonies) in question from an agar plate by means of a wire loop.

(3) Touch the loop containing the bacteria to the drop of hydrogen peroxide and observe *f*or <u>immediate</u> bubbling.

(4) Similarly test a known <u>Staphylococcus</u> specifies to verify hydrogen peroxide activity.

3-4. CITRATE TEST

a. **Principle.** This test is based on the ability of certain bacteria to utilize sodium citrate as the sole available source of carbon in a chemically defined medium.

b. **Medium.** Use Simmons citrate agar.

c. **Technique.**

(1) Lightly inoculate, by streaking only, an agar slant of Simmons citrate agar.

(2) A positive reaction (growth) is accompanied by an alkaline reaction resulting in a change in the green color of the medium to a deep blue color.

(3) No change in the indicator or the absence of blue color in the green medium indicates a negative test.

(4) A light inoculum must be used to insure that no nutrients are transferred to the chemically defined medium.

3-5. COAGULASE TEST

a. **Principle.** Coagulase activity is essentially confined to staphylococci and is related to the pathogenic species of staphylococci. Coagulase activity is demonstrated when a species of staphylococci capable of producing the enzyme coagulase is added to human or rabbit plasma and a thrombus or clot is formed. This procedure can be performed on a glass slide or in a test tube.

b. **Reagents.**

(1) Plasma, human or rabbit, fresh or dehydrated.

(2) Physiological saline (para 3-1b(2)).

c. **Slide Methods.**

(1) Place a drop of physiological saline on a clean glass slide and prepare a rather heavy, even suspension of staphylococci in the drop of saline.

(2) Place a loopful of fresh or recently reconstituted dehydrated plasma in the suspension of staphylococci. Mix and then withdraw the loop.

(3) Immediately observe for the formation of a clot. This usually occurs within a few seconds with coagulase-positive species.

d. **Tube Methods.**

(1) Transfer 0.5 ml of a 24-hour broth culture *of* staphylococci or transfer a large loopful of growth from an agar plate of staphylococci to 0.5 ml of human or rabbit plasma in a glass tube.

(2) Incubate the tube at 37º C preferably in a water bath for 3 hours. Observe approximately every 30 minutes for clotting. (Since a few strains do not clot within this period, the reading should be checked after 24 hours' incubation.)

e. **Interpretation.**

(1) Pathogenic species of staphylococci usually give a positive reaction that is evidenced when the plasma is coagulated and produces a visible clot.

(2) In negative reactions the suspension remains homogeneous and coagulation does not take place.

(3) A positive reaction, whether it be on a glass slide or in a tube is decisive. A negative slide test must be confirmed by a tube test.

3-6. FERRIC CHLORIDE TEST

a. **Principle.** This test detects the hydrolysis of sodium hippurate to benzoic acid in the presence of group B beta hemolytic streptococci. The benzoic acid combines with ferric chloride to form an observable, permanent precipitate.

b. **Reagent and Medium.**

(1) 12 percent ferric chloride. Dissolve 12 9 of ferric chloride (Fe $C1_3$) in less than 100 ml of 2 percent hydrochloric acid. Add enough 2 percent hydrochloric acid to make a total volume of 100 ml.

(2) 1 percent sodium hippurate broth,

c. **Technique.**

(1) Add 0.2 ml *of* the reagent to 0.8 ml of a 24-hour culture prepared in sodium hippurate broth.

(2) Mix immediately and observe after 5-10 minutes.

d. **Interpretation.**

(1) The formation of a permanent precipitate after approximately 10-15 minutes indicates a positive reaction.

(2) It is important to measure accurately the amounts of reagent and medium used in the test to prevent the redissolving of benzoic acid in an excess of the reagent.

(3) If evaporation *of* the broth medium occurs during incubation, replace the evaporated volume with distilled water to restore the original concentration *of* sodium hippurate.

3-7. GELATIN LIQUEFACTION TEST

a. **Principle.** Some bacteria possess the ability to produce proteolytic enzymes that can liquefy gelatin. Gelatin is a protein substance lacking essential amino acids and it is normally a liquid above 25º C.

b. **Medium.** Use ordinary extract or infusion broth containing 10 to 15 percent gelatin.

c. **Technique.**

(1) Inoculate a tube of solidified (refrigerated) nutrient gelatin by stab technique.

(2) Incubate the inoculated tube and a control gelatin tube at 37º C at which temperature the gelatin will liquefy.

(3) At the end of each 24-hour incubation period, place the inoculated tube and the control tube of liquefied gelatin in a refrigerator, for a sufficient length of time to determine whether digestion of gelatin has occurred. Check the tubes for liquefaction in this manner for as long as two weeks unless digestion occurs sooner.

d. **Interpretation.**

(1) If a portion of gelatin liquefies, this indicates a positive test.

(2) If the gelatin remains solid, this indicates a negative test.

(3) The test is frequently used to distinguish Enterobacter cloacae, which usually liquefies gelatin (delayed positive), from other members of the Enterobacteriaceae, primarily the genus Klebsiella, which is negative.

(4) Coagulase-positive staphylococci usually liquefy gelatin.

(5) An alternate procedure less frequently used is to proceed from step (1) to an incubation at 20º C at which temperature the gelatin will remain solid. In this method, the shape of the portion of gelatin being liquefied can be observed. The procedure outlined above appears to be more popular because the shape of the liquefied portion is less important than the time lost during the slower incubation at 20º C.

3-8. HYDROGEN SULFIDE TEST (LEAD ACETATE PAPER TEST)

a. **Principle.** The production of hydrogen sulfide can be detected in bacterial cultures by observing blackening of the medium that is produced when hydrogen sulfide comes into contact with certain metals, i.e., lead, iron, and bismuth, producing the sulfides of these metals. In triple sugar iron (TSI) medium, ferrous sulfate is incorporated as an indicator for hydrogen sulfide production. An alternative procedure consists of testing a growing culture for hydrogen sulfide by using lead acetate paper. The latter technique is described below.

b. **Reagent--10% Lead Acetate.** Dissolve 10 g of lead acetate $Pb(C_2H_30_2)_2 \bullet 3H_20$ in less than 100 ml of distilled water. Add enough distilled water to make a total volume *of* 100 ml.

c. **Technique.**

(1) Cut a sheet of filter paper into strips approximately 6x65 mm in size.

(2) Soak the strips of filter paper in 10 percent lead acetate solution and allow them to air-dry.

(3) Autoclave the lead acetate paper at 121º C, 15 pounds pressure, for 15 minutes in Petri dishes.

(4) Place one of the strips in the mouth of each culture tube so that a portion of the strip projects well below the cotton plug.

(5) Incubate the tubes at 37º C and examine daily to determine if blackening of the strip has occurred.

d. **Interpretation.**

(1) A positive test is indicated by blackening of the strip.

(2) No evidence of blackening of the strip indicates that the test reaction is negative.

(3) The filter paper technique is especially useful for detection of Brucella species and for distinguishing among the species of Brucella. When an attempt is made to distinguish among the Brucella species, the filter paper strips must be changed daily after the first appearance of blackening.

3-9. INDOLE TEST

a. **Principle.** This test is based on the ability of certain bacteria to split the amino acid tryptophan into alanine and indole. The liberated indole combines with paradimethyaminobenzaldehyde in Kovac's reagent to give a deep red color.

b. **Reagents and Media.**

(1) Kovac's reagent. Dissolve 5 g of paradimethyaminobenzaldehyde (chemically pure) in 75 ml of amyl or butyl alcohol. Add enough concentrated hydrochloric acid (HCl) to make a total volume of 100 ml.

(2) Tryptophan broth OR Trypticase nitrate broth.

c. **Technique.**

(1) Add 0.5 ml of Kovac's reagent to 3 to 5 ml of a 24 to 48 hour broth culture of the organism.

(2) Observe for the appearance of a red color at the interphase between the reagent and the broth culture.

d. **Interpretation.**

(1) The presence of the red color at the interphase between the reagent and the broth culture signifies that indole has been liberated.

(2) The absence of the red color signifies a negative test.

3-10. METHYL RED TEST

a. **Principle.** When they ferment glucose, some organisms produce a small quantity of acids that are then converted to neutral end products. This test is designed to differentiate those organisms which, in contrast, produce a high acidity.

b. **Reagent and Medium.**

(1) Methyl red indicator. Dissolve 0.02 g of methyl red in 50.0 ml of 95 percent ethyl alcohol and add 50.0 ml of distilled water.

(2) MR-VP (methyl red-Voges-Proskauer) broth (Clark and Lubs medium).

c. **Technique.** Add about 5 drops of methyl red indicator to a 5 ml portion of a 2 to 7 day-old culture, prepared in MR-VP broth.

d. **Interpretation.**

(1) A positive reaction occurs when the culture is sufficiently acid to turn the methyl red indicator to a distinct red color.

(2) A yellow color indicates a negative test.

3-11. NITRATE REDUCTION TEST

a. **Principle.** Bacteria are grown in a broth culture containing potassium nitrate to determine the ability of the bacteria to reduce nitrate to nitrite.

b. **Reagents and Medium.**

(1) Solution A. Dissolve 8 g of sulfanilic acid in less than 1,000 ml of 5N acetic acid (this consists of 1 part of glacial acetic acid and 2.5 parts of distilled water). Add enough 5N acetic acid to make a total volume of 1,000 ml.

(2) Solution B. Place 6 ml of N,N,-dimethyl-1-naphthy-lamine, in a 1-liter flask and fill to the 1,000-ml mark with 5N acetic acid. CAUTION: Avoid aerosols, mouth pipetting, and contact with skin due to possible carcinogenicity. (A similar chemical, alpha-naphthylamine, is listed as a carcinogen.)

(3) Zinc dust.

(4) Mix 5 g of tryptone, 5 g of neopeptone, and 1,000 ml of distilled water. Boil and adjust the pH to 7.3-7.4. Add 1 g of potassium nitrate (reagent grade) and 0.1 g of glucose. Dispense 5 ml per tube and sterilize at 121^{O} C, 15 pounds pressure, for 15 minutes.

c. **Procedure.**

(1) Add 1 ml of solution A to approximately 5 ml of a 24-hour culture grown in nitrate broth.

(2) Add, dropwise, 1 ml of solution B and observe for a light pink to deep red color.

d. **Interpretation.**

(1) A light pink to deep red color is a positive result. Nitrate has been reduced to nitrite by the bacteria.

(2) The absence of color is an equivocal result. Add a small amount of zinc dust to the colorless mixture of culture and reagents.

(a) If the solution is colorless after the addition of zinc, the result is positive. Nitrates have been reduced first to nitrites and then further reduced.

(b) If the solution is red after the addition of zinc, the result is negative. Nitrates have been reduced by the zinc but not by the bacteria.

3-12. OXIDASE TEST

a. **Principle.** This test detects the enzyme oxidase produced by members of the genus Neisseria and the genus Pseudomonas. Oxidase reacts with the reagent to produce a colored compound.

b. **Reagent.** Add 0.1 g of tetramethyl-p-phenylenediamine hydrochloride to 10 ml of distilled water. Allow to stand for 15 minutes before using, but use within 2 hours or it will begin to lose its activity.

c. **Technique.**

(1) Place 2 to 4 drops of reagent on a strip of filter paper.

(2) Remove a portion of the colony with a sterile platinum loop. (Iron-containing wire may give a false-positive reaction.)

(3) Rub the portion of the colony on the impregnated paper.

(4) A positive reaction occurs if the moist paper near the bacteria turns dark purple within 10 seconds.

d. **Alternate Method.**

(1) Use a 1 percent solution (0.1 g in 10 ml) of dimethyl-p-phenylenediamine hydrochloride with the same time restrictions stated in b above. This reagent is less sensitive and more toxic than the one above.

(2) Place 2 to 3 drops of suspected colonies of the culture plate.

(3) Oxidase-positive colonies develop a pink color that gradually turns maroon, then dark red, and finally black.

e. Note that the oxidase test does not interfere with the gram reaction. A gram stain must be performed on all oxidase-positive colonies; gram-negative diplococci that are oxidase positive may be tentatively identified as Neisseria.

3-13. UREASE TEST

a. **Principle.** The splitting of urea to ammonia and carbon dioxide by the enzyme urease may be employed to help differentiate Proteus species from other members of the enteric group. Proteus species and other organisms hydrolyze urea and release ammonia, which is indicated by a reddening of the indicator phenol red, included in the medicine.

b. **Medium.** Use either urea agar (Christensen) slants or urease test broth (Rustigian and Stuart).

c. **Technique.**

(1) Transfer a heavy inoculum to the urea medium. (The broth should be shaken to suspend the bacteria.)

(2) Incubate at 37º C.

(a) Agar slants should be observed at 2 hours, 4 hours, and after overnight incubation. Negative tubes should be observed daily for 4 days for delayed reactions due to non-Proteus species.

(b) Broth cultures should be read after 10 minutes, 60 minutes, and 2 hours. Longer incubation is required for non-Proteus species.

d. **Interpretation.** Proteus species give a red color, a positive test in 2 to 4 hours. Other urease-positive organisms produce a red color much more slowly. If no red color is produced, the test is negative.

3-14. VOGES-PROSKAUER (VP) TEST

a. **Principle.** This test detects the production of the neutral end product acetylmethylcarbinol during fermentation of dextrose. A positive test, a red color, is characteristic of Enterobacter cloacae, Enterobacter aerogenes, and Klebsiella species. A negative test is characteristic of E. coli, Shigella, and Salmonella.

b. **Reagents and Medium.**

(1) Five percent alpha-naphthol. Dissolve 5g of alpha -naphthol in less 100 ml of absolute ethyl alcohol. Add enough absolute alcohol to make a total volume of 100 ml.

(2) Forty percent potassium hydroxide (K0H). Because of its hygroscopic nature, 40 g of potassium hydroxide must be rapidly weighed. Dissolve in less than 100 ml of distilled water. (The container may be placed in a sink with cold circulating water to control temperature.) Cool and add sufficient distilled water to make a total volume of 100 ml.

(3) MR-VP (methyl red-Voges-Proskauer) broth (Clark and Lubs medium).

c. **Technique.**

(1) Add 0.6 ml of 5 percent alpha-naphthol in absolute ethyl alcohol to 1 ml of a 24 to 48 hour broth culture prepared in MR-VP broth.

(2) To the same culture, add 0.2 ml of 40 percent potassium hydroxide.

(3) Shake the tube and allow it to stand for 5 to 10 minutes before interpreting results.

d. **Interpretation.**

(1) A positive test is indicated by the development of a pink or red color.

(2) A yellow color is regarded as a negative.

(3) Occasionally, some difficulty is experienced in obtaining a positive VP result with organisms that should produce acetylmetholcarbinol. This may be overcome by gently heating the culture after adding the test reagents. Strains that do not produce acetylmethylcarbinol (VP-negative) will still yield a negative result despite the application of gentle heat.

Section II. GRAM-POSITIVE COCCI: STAPHYLOCOCCI

3-15. GENERAL COMMENTS ABOUT GRAM-POSITIVE COCCI

The organism comprising the gram-positive cocci are chiefly the staphylococci, streptococci, and pneumococci. Collectively, these organisms are responsible for a variety of human infections. Such infections range from relatively simple involvements of the skin and mucous membranes to more serious diseases that may be manifested in pneumonia, septicemia, rheumatic fever, acute glomerulonephritis, or deep tissue abscesses. An identification schema for aerobic gram-positive bacteria is provided in figure 3-1.

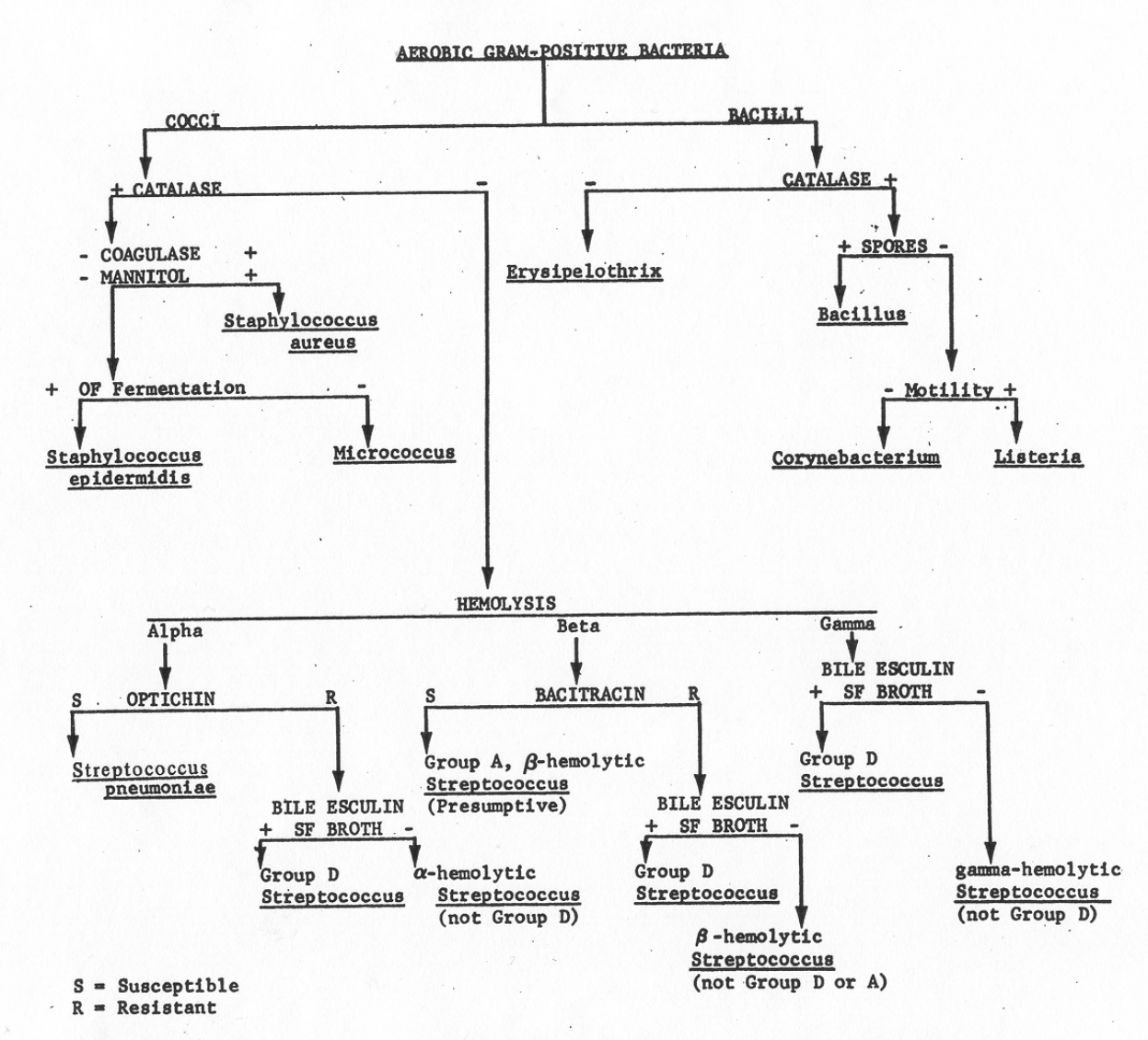


Figure 3-1. Identification schema for aerobic gram-positive bacteria.

3-16. PATHOGENICITY OF STAPHYLOCOCCI

a. **Common Manifestations.** The staphylococci (figure 3-2) are ubiquitous in nature. They occur as normal inhabitants on the skin and in the respiratory and gastrointestinal tracts of man. The majority of such forms are the comparatively avirulent organisms. Staphylococcus epidermidis and the related forms of Micrococcus and Sarcina are saprophytes frequently isolated from the skin and mucous membranes. Staphylococcus aureus strains are usually responsible for the staphylococcal diseases of man. These forms occur especially in the upper respiratory tract of asymptomatic individuals. The asymptomatic carrier is of considerable importance in transmitting these organisms. Staphylococcal diseases are most commonly manifested in localized suppurations that may be in the form of simple pustules, hair follicle infections, boils, or extensive carbuncular conditions that may progress to form metastatic abscesses in any tissue. The latter results from the spread of the organism via the blood stream. A majority of cases of osteomyelitis, enterocolitis, otitis media, and sinusitis are of staphylococcal etiology. Pneumonia, meningitis, and endocarditis are relatively infrequent manifestations of the staphylococci.

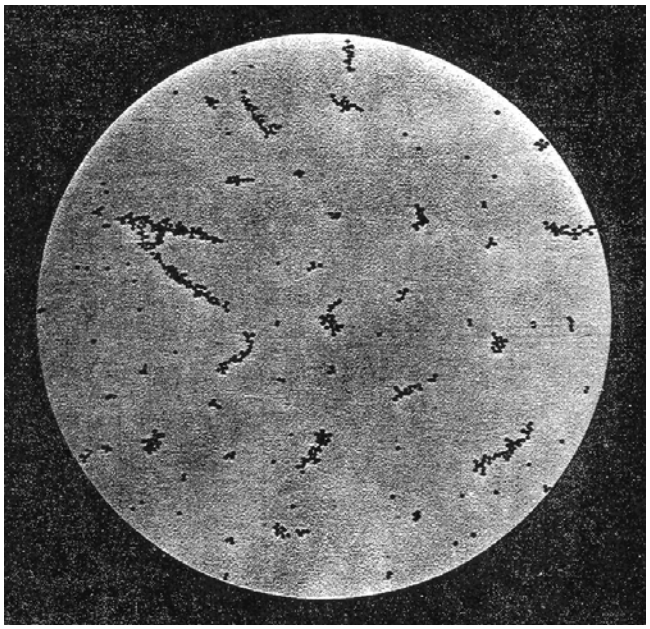


Figure 3-2. Gram-stained smear of Staphylococcus species from broth culture.

b. **Enzyme Secretions.** The virulent staphylococci excrete a variety of substances that account for their ability to invade tissue and cause disease in man. Coagulase is an enzyme produced by pathogenic staphylococci. Coagulase causes a clotting of plasma which results in the formation of a layer of fibrin around a given staphylococcal lesion. Although this fibrin wall may confine the infection to a localized process, it also serves as a protective barrier for the organism against phagocytic activity and the action of antimicrobial drugs. This, then, is one of the very valuable clinical laboratory tests used to identify Staphylococcus aureus.

c. **Toxic Production.** A variety of toxins may appear in cultures 1 to 3 days old when grown in a semisolid infusion agar of veal or beef. These cultures must be incubated in an atmosphere of 20 to 40 percent carbon dioxide. The exotoxins may be destroyed by heating to 55º to 60º C. Any of the following may be present in such a filtrate: lethal toxin, hemolysin, leukocidin, and dermonecrotic toxin.

d. **Hospital Staphylococci.** Staphylococci have eventually become resistant to practically every antibiotic introduced to combat its presence. Each succeeding generation seems to be more resistant to drugs than the previous parental strain. This has been primarily due to the promiscuous use of antibiotics to treat everything from a cold to a sore toe. Since the advent of penicillin, when there were almost no drug-resistant strains, certain staphylococcus species have been particularly adept at developing drug resistance. Since antibiotics were originally administered in low doses for such widely divergent ailments, those strains of organisms which were not eliminated by use of drugs have been able to develop into strains which are particularly pathogenic and resistant to almost every antibiotic that has been developed. This has led to the necessity of determining a series of tests to discover which antibiotic is effective for each patient. The antibiotic that is more effective against one strain of cocci for one person may not be best for another person with a different strain. Some larger laboratories perform phage tying of hospital staphylococci for epidemiologic purposes.

3-17. CULTURAL CHARACTERISTICS OF STAPHYLOCOCCI

a. Staphylococci are nommotile, nonsporogenous, and usually do not form capsules. The exception to capsule formation is in very young broth cultures after a few hours' incubation. These bacteria always stain gram-positive. Those cocci not staining gram-positive are due to old and dying cultures, organisms phagocytized by white cells, and those in the center of clusters.

b. The staphylococci grow readily on ordinary nutrient media without the presence of special enrichments. The more commonly occurring species are facilitative organisms. A few staphylococci, which may be weakly pathogenic, are strict anaerobes. The staphylococci isolated from human disease grow well at 37º C. Abundant growth usually takes place in 18 to 24 hours of incubation.

c. The colony morphology of Staphylococcus species is usually characteristic. After 1.8 hours of incubation at 37º C on an agar media, staphylococci form rather large colonies ranging from 2 to 4 mm in diameter. The colonies are opaque, round, smooth, raised, glistening and with an entire (even) margin. The colonies may develop a characteristic golden, porcelain-white, or lemon-yellow pigment. The colony is soft or "butter-like" in consistency.

d. Pigmentation is more evident after plates of the cultivated organism have been exposed to room temperature overnight. Young colonies of staphylococci are not pigmented and appear colorless. As growth continues, a pigmentation results which will not diffuse into the surrounding medium. This is what gives pus and sputum a faint golden yellow color and usually indicates possible infection with staphylococci. All of the characteristics given up to this point relate to the genus Staphylococci.

e. Colonies of Staphylococcus aureus typically have a golden pigmentation on initial isolation while colonies of Staphylococcus epidermidis are usually white when cultivated on blood agar. When grown on blood agar, Staphylococcus aureus usually causes beta hemolysis (complete lysis of the RBC's), resulting in a clear zone around the colony, while Staphylococcus epidermidis generally does not hemolyze the RBC's.

3-18. LABORATORY INDENTIFICATION OF STAPHYLOCOCCI

a. It must be emphasized that on the basis of gram morphology Staphylococcus aureus and Staphylococcus epidermidis cannot be differentiated; however, the genus can usually be established on the basis of gram stain morphology. Staphylococcus species are gram-positive. Members of the genus Staphylococcus are spherical or oval cocci measuring approximately 1 micron in diameter. Most species are typically arranged in irregular clumps resembling grapelike clusters. In broth cultures in particular, single cocci, pairs, and occasional short chains may also be seen. Related organisms belonging to the genus Gaffkya and the genus Sarcina are morphologically similar to the staphylococci except that: they usually occur in uniform groups of four and eight cells respectively.

b. Inoculation and incubation of blood agar would reveal the typical colony morphology. Again, no species differentiation is possible since hemolytic and pigmentation patterns are variable.

c. A medium that is especially useful when working with clinical specimens suspected of containing Staphylococcus species is mannitol salt agar (MSA). This medium contains 7.5 percent sodium chloride, and this concentration of salt is inhibitory to most bacteria. Staphylococcus species are halophilic (salt-loving), and are therefore able to grow on MSA. The medium also contains the carbohydrate mannitol that is usually fermented by Staphylococcus aureus, thus yielding acid conditions. Staphylococcus epidermidis does not usually ferment this sugar. The indicator phenol red is also incorporated into this medium. Under acid conditions, phenol red will give a yellow color; under alkaline conditions, a red color will result. Staphylococcus aureus usually ferments mannitol with the development of a yellow color in the medium around the colonies. Staphylococcus epidermidis does not usually ferment mannitol, and the indicator imparts a redder shade to the medium.

d. The most important differentiating test for species identification is the coagulase test. This test positively differentiates the pathogenic Staphylococcus aureus from the nonpathogenic Staphylococcus epidermis. In terms of result, Staphylococcus aureus is coagulase positive while Staphylococcus epidermis is coagulase negative.

3-19. CONFIRMATORY PROCEDURES

To establish identity, select an isolated Staphylococcus-like colony and proceed with the following studies.

a. **Gram Stain Smear.** Prepare gram-stained smears and examine for the typical gram-positive cocci in grape-like clusters.

b. **Catalase Test.** Perform the catalase test (Lesson 5) to distinguish staphylococci from streptococci. Staphylococci produce the enzyme catalase, which when mixed with hydrogen peroxide will liberate oxygen from the hydrogen peroxide to produce vigorous bubbling. Immediate bubbling in the drop of hydrogen peroxide signifies a positive test. Staphylococci are catalase positive. Absence of immediate bubbling signifies a negative test. Streptococci and pneumococci are catalase negative. If the loop digs into blood agar medium, blood cells from blood agar may produce a false-positive reaction.

c. **Coagulase Test.** Pathogenic species of staphylococci usually give a positive reaction that is evidenced when the plasma is coagulated and produces a visible clot. In negative reactions the suspension remains homogeneous and coagulation does not take place. A positive reaction, whether it be on a glass slide or in a tube is decisive. A negative slide test must be confirmed by a tube test. See Lesson 5 for details.

d. **Mannitol Salt Agar.** Inoculate a mannitol salt agar plate and observe for growth and fermentation of the mannitol at 18 to 24 hours of incubation at 37º C. If the mannitol is fermented, the plate will turn from a reddish-pink color to yellow.

e. **Liquefaction.** Inoculate a tube of gelatin by stabbing some growth into the medium. Incubate at room temperature for 3 to 5 days and observe for liquefaction

f. **Primary Means of Distinguishing S. aureus from S. epidermidis.** Although pigmentation, hemolysis, and gelatin liquefaction are all demonstrable features of most Staphylococcus aureus strains, the index of their pathogenicity is coagulase production and mannitol fermentation.

Section III. GRAM-POSITIVE COCCI: STREPTOCOCCI

3-20. INTRODUCTION TO THE STREPTOCOCCI

a. The streptococci (figure 3-3) are spherical to ovoid cocci ranging between 0.8 and 1.0 micron in diameter. The cocci predominantly occur in chains; however, paired or single cells may also be observed. Characteristic chains are more typical in smears from broth cultures. The streptococci are nommotile and nonsporeforming. They are typically stained gram-positive, although gram-negative forms are occasionally observed in specimens of old cultures.

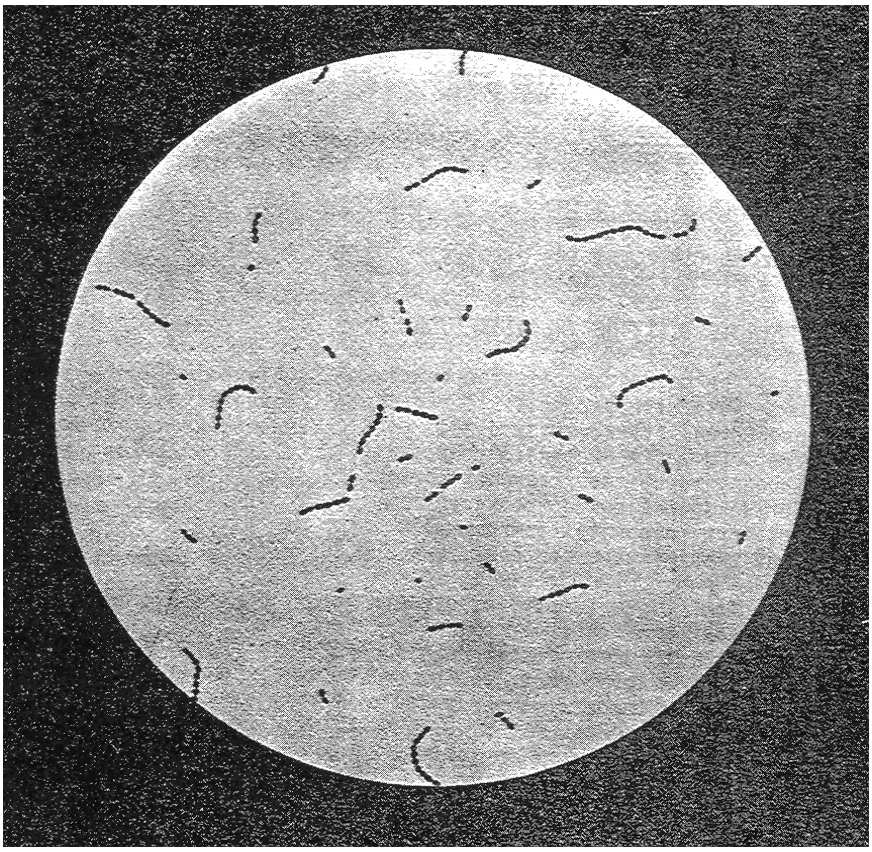


Figure 3-3. Gram-stained smear of Streptococcus species from broth culture.

b. Certain Streptococcus species produce polysaccharide capsules, while others produce a capular substance composed of hyaluronic acid. The presence or absence of capsules is not a distinct feature for use in routine identification of streptococcal forms.

c. Several schemes for classifying the streptococci have been devised. The Lancefield classification has as its basis the antigenic structure of the organisms. The structure of a carbohydrate antigen ("C" substance) is different for each group in this series. The groups are designated by the letters A through O. According to this classification, group A strains are the most common human pathogens.

d. A second classification, devised by Sherman, has as its basis both the physiologic and immunologic characteristics of the: streptococci. The Sherman classification is composed of the pyogenic streptococci, the viridans group, the enterococci, and the lactic streptococci. The human pathogens are in the pyogenic group.

3-21. HEMOLYTIC PATTERNS

The most useful method for preliminary differentiation of streptococci is the pattern of hemolysis on blood agar.

a. The hemolytic action of Streptococcus species is influenced by the type of blood used in blood agar. The blood of choice for study of hemolysis is defibrinated sheep blood in a concentration of 5 percent. Glucose should be excluded since it may obscure hemolytic reactions. If sheep blood is not available, use rabbit or horse blood.

b. The pour plate method (see Lesson 2, Section II) is by far the best method for studying hemolytic activity; it provides subsurface colonies of streptococci whose hemolytic activity is not greatly affected by oxygen. For convenience, a general practice is to study the hemolytic patterns of surface colonies. It must be remembered, however, that the hemolytic activity of such surface colonies may not be completely typical.

c. The four types of hemolytic patterns, which are best observed under low power (100X) magnification, are defined as follows:

(1) Alpha. An indistinct zone around a colony in which the red cells are partially destroyed. There is often a greenish or brownish discoloration of the medium near the colony.

(2) Beta. A clear, colorless zone around a colony that indicates complete lysis of the red cells. This is best seen in deep colonies in a pour plate since oxygen affects the activity of hemolysins and may make surface colonies appear to be alpha or nonhemolytic.

(3) Gamma. No apparent hemolytic activity or discoloration around the colony.

(4) Alpha prime or wide zone alpha. A small halo of intact or partially lysed cells immediately surrounding the colony, with a zone of complete hemolysis extending further into the medium. Without a microscope, this can be composed with beta hemolysis.

3-22. PATHOGENICITY OF THE STREPTOCOCCI

a. The majority of streptococcal infections of man are caused by beta hemolytic streptococci. A variety of diseases are manifested such as: puerperal fever, erysipelas, septic sore throat scarlet fever, impetigo and acute bacterial endocarditis. Of these infections, septic sore throat is, by far, the most common clinical entity. Approximately 2 to 3 weeks following recovery from a beta streptococcal pharyngitis, acute glomerulonephritis or rheumatic fever may develop not as a direct effect of disseminated bacteria, but due to tissue hypersensitivity.

b. The alpha hemolytic streptococci, especially those that are normal inhabitants of the upper respiratory and intestinal tracts, can cause disease if normal resistance is reduced. Alpha hemolytic members from the respiratory tract may cause subacute bacterial endocarditis, and Group D streptococci commonly cause urinary tract infections.

c. The anaerobic streptococci commonly encountered in the normal vaginal flora, in the mouth, and in the intestine are capable of giving rise to suppurative lesions. Infections of these organisms produce pus with a foul odor.

d. The nonhemolytic streptococci are practically all saprophytic forms that have been isolated from milk and various dairy products. A few strains have been implicated as causing subacute bacterial endocarditis (SBE).

3-23. CULTURAL CHARACTERISTICS OF THE STREPTOCOCCI

Culturally, streptococci grow on rich, artificial media. For primary isolation, use blood peptone agar or peptone agar with blood serum or ascitic fluid for best results. The addition of 0.5 percent glucose increases growth but interferes with hemolysis. An ordinary meat infusion medium, such as nutrient agar or broth, will generally not support the growth of Streptococcus species. The standard blood agars and' such broth media as thioglycollate, Todd-Hewitt, and Trypticase soy are successfully used in cultivating most species. The majority of the streptococci are facultative anaerobes, although some strains, especially those forms isolated from deep tissue infections, are obligate anaerobes. Growth is best at 37º C for pathogenic, hemolytic forms. After 24 hours' incubation on peptone blood agar, the streptococci generally appear as small (approximately 1 mm or less), slightly granular, circular, convex, translucent colonies. Some streptococci give rise to matt or mucoid colonies on primary isolation. Such colonies possess M protein antigen. After repeated transfers on laboratory media, rough or smooth colonies all eventually develop which are avirulent and void of M protein. Restoration of M protein and virulence to rough or smooth forms may be accomplished by repeated, rapid animal passage.

3-24. GENERAL IDENTIFICATION STREPTOCOCCI

Gram-stained smears of throat and nasopharyngeal materials and sputum are of limited value since saprophytic streptococci are usually present. Smears of spinal fluid are of definite value in revealing gram-positive cocci predominantly in chains. Smears of urinary sediments of exudates from localized lesions, wounds, or deep abscesses (peritonitis) may enable intelligent selection of culture procedures. The streptococci are such a heterogeneous group of organisms that neither morphological, physiological, nor immunological studies alone have been completely successful in classification of all species encountered. Hemolytic reaction is the criterion upon which streptococci are classified and reported. Clinical evidence coupled with the savings in resources supports the hemolytic criterion as being both practical and acceptable. The type of hemolysis exhibited on blood agar (alpha, beta, or gamma) affords tentative separation of streptococcal species. In all cases, suspected colony types should be verified as being streptococci by gram-staining. The catalase test is useful in differentiating culturally similar beta hemolytic streptococci and hemolytic staphylococcal forms. Streptococci are catalase negative.

3-25. BETA HEMOLYTIC STREPTOCOCCI

The beta hemolytic streptococci are identified on the basis of cultural and physiological characteristics. Table 3-1 lists some significant biochemical reactions that will usually enable identification of those groups responsible for the majority of human infections.

a. **Bacitracin Susceptibility.** A useful means for presumptive identification of group A, beta hemolytic streptococci uses a 0.04-unit bacitracin disk. The following precautions are advised.

(1) Be sure the bacitracin disk is manufactured for differential studies, not sensitivity studies.

(2) Use a heavy inoculum of a pure culture.

(3) Be sure the colony is beta hemolytic.

(4) Periodically check commercial disks with control strains.

(5) Any zone of inhibition is positive.

b. **Sodium Hippurate Broth.** Sodium hippurate broth is used to enable differentiation of group B streptococci from groups, A, C, and D. The medium is inoculated with the unknown and incubated for 48 hours. The broth is then tested for the possible presence of benzoic acid formed by the organism's hydrolysis of sodium hippurate.

	GROUP A	GROUP B	GROUP C (Human)	GROUP D (Enterococci)
Surface colonies blood agar	Greyish-white, opaque to translucent; hard, with tendency for whole colony to move on probing; hemolytic zones approximately 2 mm.	Grey translucent, with soft texture; slight hemolytic zone.	Indistinguishable from group A.	Grey, somewhat translucent, soft; hemolytic zones slightly wider than the colonies.
Subsurface colonies blood agar	Lens or disc shaped with 2-mm zone of hemolysis.	0.5-mm zones of hemolysis after 24 hours; 0.1-mm zone after 48 hours. Double ring hemolysis following refrigeration overnight.	Indistinguishable from group A.	Hemolytic zones approximately 3-mm.
SF medium	No growth	No growth	No growth	Growth, with acid reaction.
Sodium hippurate	Negative	Positive	Negative	Negative
Bile esculin medium	Negative	Negative	Negative	Positive
Bactracin susceptibility	Susceptible	Resistant	Resistant	Resistant
6.5 percent NaCl broth	No growth	No growth	No growth	Growth

Table 3-1. Identification of beta hemolytic streptococci (continued).

	GROUP A	GROUP B	GROUP C (Human)	GROUP D (Enterococci)
Source	Human infections and carriers (usually respiratory).	Bovine infections and milk. Human intestinal, genitourinary, and respiratory tracts.	Upper respiratory and intestinal tracts of humans.	Intestinal and genitourinary tracts of humans. Milk and milk products.
Disease	Erysipelas, impetigo, septic sore throat, scarlet fever, rheumatic fever, acute glomerulonephritis, puerperal sp.	Urinary tract infections, peritonitis endometritis, wound infections, rarely septicemia.	Suppurative lesions of man, usually in mixed infections. Throat infections.	Same as group B.

Table 3-1. Identification of beta hemolytic streptococci (concluded).

c. **SF Broth.** When dealing with a beta Streptococcus species, a most important laboratory procedure is that of determining whether the organism is an enterococcus (group D). This is successfully accomplished using SF (Streptococcus faecalis) broth. SF broth is selective in that only Streptococcus faecalis and other members of group D streptococci are able to grow in it. The concentration of sodium azide in this medium will prevent the growth of other bacteria. The suspected pathogenic Streptococcus species should be inoculated to SF broth from a pure colony. After 24 hours' incubation at 37º C, the broth is examined for the presence or absence of growth. Visible growth is observed, coupled with an indicator change from purple to a yellowish brown.

d. **Bile Esculin Medium (BEM).** Considered to be more reliable than SF broth, the test with bile esculin medium is recommended for presumptive identification of group D streptococci. BEM contains both bile (oxgall) and esculin. It is selective for growth of group D streptococci (also Enterobacteriaceae and Listeria monocytogenes). When esculin is hydrolyzed, a dark brown or black color results. The medium is inoculated with a pure culture and incubated for 48 hours at 37º C. A brownish color is positive.

e. **Infusion Broth With 6.5 Percent.** This medium is selective for Group D enterococci. (Group D streptococci that are not enterococci will not grow.)

3-26. DIFFERENTIATION OF OTHER STREPTOCOCCI

Alpha and gamma streptococci, other than enterococci, are usually differentiated on the basis of colonial characteristics and reactions obtained from biochemical media. From a practical standpoint, it is only necessary to differentiate Streptococcus faecalis from other gamma and alpha streptococci. This is necessary because Streptococcus faecalis is usually resistant to penicillin, streptomycin, and sulfonamides, while the other gamma and alpha strains are sensitive to these therapeutic agents. It is also important to differentiate Streptococcus pneumoniae from alpha streptococci. Since pneumococcal strains form green hemolytic colonies similar to colonies of alpha streptococci on blood agar, it is necessary to employ the bile solubility test and other studies for their distinction.

Section IV. GRAM-POSITIVE COCCI: PNEUMOCOCCI

3-27. MORPHOLOGY OF PNEUMOCOCCI

The pneumococci (Streptococcus pneumoniae, formerly Diplococcus pneumoniae are small, slightly elongated cocci arranged in pairs (diplococci). In young cultures or specimens the diplococci are frequently lancet-shaped on each end with adjoining sides flattened or slightly curved. The organisms (figure 3-4) may occur singly or in short chains. The pneumococci are typically gram-positive but rapidly become gram-negative with the tendency to lyse spontaneously (autolysis). The organisms are nommotile and nonsporeforming. Virulent pneumococcal cells are enveloped with a well-defined polysaccharide capsule that is prominent when the organisms are observed in tissue exudates. The capsules of avirulent pneumococci are less demonstrable. When appropriately stained, the capsule normally appears as a clear halo around the cells.

3-28. GROWTH REQUIREMENTS OF PNEUMOCOCCI

a. In terms of growth requirements pneumococcal strains are fastidious. Enriched media is need for cultivation, and blood agar is recommended as the isolation medium. The optimum temperature for growth is 37º C. Although the pneumococcus is classified as a facultative anaerobe in regards to its oxygen requirements, growth is enhanced by increased CO_2 tension. This increased CO_2 tension is readily supplied by use of a candle jar or CO_2 incubator.

b. Animals may be used for isolation and determination of pathogenicity of pneumococci. White mice are injected intraperitoneally with the sample. This is the most rapid and reliable method for obtaining pure cultures of the pneumococci, and for determining strain pathogenicity. It is possible to use Avery's artificial mouse in place of live animals for easy isolation of pneumococci. The artificial mouse is a meat infusion broth that includes 1 percent glucose and 5 percent defibrinated rabbit blood with a final pH of 7.8. Hiss capsule stain of Streptococcus pneumoniae from initial isolation in broth culture.

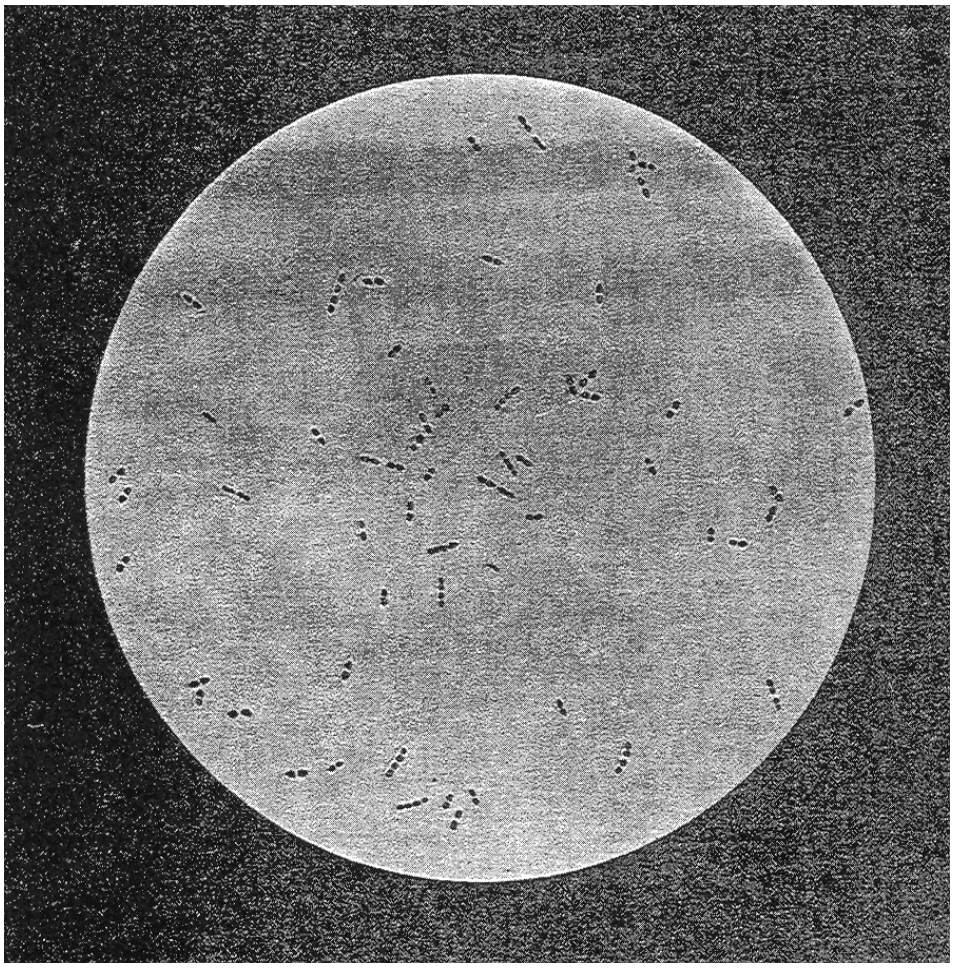


Figure 3-4. Hiss capsule stain of Streptococcus pneumoniae from initial isolation in broth culture.

3-29. COLONY CHARACTERISTICS OF PNEUMOCOCCI

a. On blood agar, after 18 to 24 hours' incubation, typical virulent strains or pneumococci form smooth, flat, transparent, slimy or mucoid colonies (0.5-1.5 mm in diameter), which may possess a central depression and ringed periphery. The colonies are alpha hemolytic. They resemble the other alpha hemolytic streptococci, but the pneumococci are usually more flattened and moist. In peptone broth there is rapid growth in 24 hours, with a faint, uniform clouding of the fluid. Growth is improved by the addition of 2 to 4 percent peptone, or adding 1 part blood serum or ascitic fluid to 3 parts broth.

b. The majority of pathogenic pneumococci are encapsulated, which accounts' for the formation of smooth or "S" colony types. The nonencapsulated organisms may become the predominating cells when the organisms are cultivated on an agar medium containing their respective type-specific antipolysaccharide serum. Nonencapsulated cultures developed in this manner exhibit rough or "R" type colonies. Loss of the capsule from the pneumococcal strain is accompanied by a loss of virulence and antigenic (type) specificity since the capsular substance influences these capabilities.

3-30. PATHOGENICITY OF PNEUMOCOCCI

a. **Disease Manifestations.** Pneumococci are responsible for approximately 80 percent of the cases of lobar pneumonia and roughly 1.5 percent of bronchial pneumonia in man. These infections are characterized by sudden onset, with accompanying chills, fever, and sharp pleural pain. Sputum from affected individuals is usually bloody or rusty; possessing a thick, viscous consistency. In the early stages of pneumococcal pneumonia, bacteremia may be present. Pneumococci may infect other tissues, either as complications of pneumonia or as independent and primary infections. From the respiratory tract, the organisms most frequently spread to the sinuses and middle ear. Meningitis may result from blood stream infection following pneumonia. Localized infecti'Ol'1s may occur in any part of the body. Virulent pneumococci are generally spread by asymptomatic carriers. The pneumococci are opportunist pathogens in that they apparently do not invade except when an individual's general resistance is lowered. Pneumococcal pneumonia has hastened the fatal termination of such diseases as viral influenza, tuberculosis, congestive heart failure, and cancer. The virulence of these organisms is in part dependent upon the capsule, which functions as a barrier against ingestion by phagocytes in the animal host.

b. **Neufeld Quellung Reaction.** Various pneumococcus strains possess antigenically distinct polysaccharide capsules, each inducing the formation of specific antibodies in the serum of animals upon tissue contact. The antipolysaccharide antibodies are specific, in that once they are formed, they will react typically with only the particular type of capsular polysaccharide that initiated their production. Such reactions result in destruction and removal of the capsule from the cell wall, thereby rendering the pneumococcus vulnerable to phagocytosis. This characterizes the immune state possessed by individuals following recovery from pneumococcal infections. Variations in the molecular makeup of capsular polysaccharides throughout the pneumococci amount for 84 known immunologically distinct pneumococcal types. The various types have been determined by animal inoculation with purified capsular extracts of pneumococcal cells (specific soluble substance, or SSS) and subsequent demonstration of specific antibody formation in the animal's serum. Pneumococcal types are arbitrarily designated by Roman numerals or lower case letters. The serological identification of pneumococci is accomplished in vitro using slide tests that entails exposing the organisms to type-specific antisera against the strains most commonly responsible for human infections. When a particular pneumococcus type is mixed with its specific antipolysaccharide antibody on a glass slide, the capsule appears to swell and becomes markedly defined upon microscopic observation. This is known

as the quellung reaction; however, it is not used as a routine test for the identification of the pneumococci.

3-31. LABORATORY IDENTIFICATION OF PNEUMOCOCCI

a. Gram-stained smears of sputum specimens may reveal the typical gram-positive, lancet-shaped diplococci. The organisms may also be observed in spinal fluid sediments, pleural fluids, or other exudates from infected tissue. The presence of capsules may be noted in gram-stained smears as thick halos around the cells when observed under reduced light. Demonstration of the capsule is better accomplished by preparing a capsule stain or an India ink preparation as previously discussed.

b. On blood agar plates pneumococcal colonies are generally well formed after 18 to 24 hours' incubation in a candle jar at 37º C. Plates should not be discarded before 48 hours' incubation time since primary growth to certain types develops slowly. Colony growth may be obtained only on blood agar plates streaked from primary broth cultures.

c. Although typical colonies of pneumococci are rather distinctive, the growth cannot be grossly distinguished with certainty from that of alpha hemolytic streptococci. No report should be rendered as to the presence of pneumococci or alpha streptococci without performing confirmatory studies.

3-32. CONFIRMATORY STUDIES

Isolated colonies suggestive of pneumococcus (table 2-3) should be examined as follows.

a. **Gram Stain Smears.** Prepare gram-stain smears and examine for gram-positive diplococci that may be lancet-shaped.

b. **Bile Solubility Test.** Perform the bile solubility test (Section I) and observe for dissolution of growth. Pneumococci are lysed in the presence of bile salts; other streptococci are not lysed. This test is used to distinguish between the alpha hemolytic streptococci and pneumococci, which are also alpha hemolytic. If the organism is Streptococcus pneumoniae, clearing should occur in the test tube containing the sodium deoxycholate within the 3-hour incubation period. Other alpha hemolytic streptococci cultures remain turbid like the control.

c. **Optochin Sensitivity Test.** Inoculate a blood agar plate for determination of Optochin sensitivity. The Optochin sensitivity test may be performed in lieu of the bile solubility test. Optochin (ethylhydrocupreine hydrochloride) discs are placed on the heavily inoculated plate. After 18 to 24 hours' incubation, pneumococcal growth will exhibit a large, clear zone of inhibition. The results of the bile solubility test (or the Optochin sensitivity test) along with careful observation of colonies and microscopic studies are usually sufficient criteria for identification of pneumococci.

Organism	Hemolysis	Colonial Morphology	Capsule	Quelling Reation In Type-Specific Antiserum	Solubility In Sodium Deoxcholate	Optochin Sensitivity
Pneumoncocci	Alpha	Small, flat shiny or mucoid colonies with concentric rings and depressed centers.	Present	Reactive	Soluble	Sensitive
Alpha Streptococci	Alpha	Small, raised, dome-shaped, smooth colonies which are translucent or opaque.	Absent	Nonreactive	Insoluble	Insensitive

Table 3-2. Differentiation of pneumococcal strains from alpha streptococci.

Continue with Exercises

EXERCISES, LESSON 3

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. Unlike other alpha hemolytic streptococcus, pneumococci are lysed in a solution of:

   a. Sodium chloride.

   b. Sodium hydroxide.

   c. Sodium deoxycholate.

   d. Hydrogen peroxide.

2. In the bile indicated bile solubility test, the presence of pneumococci is indicated by _____ in the tube labeled test.

   a. Clearing.

   b. Turbidity.

   c. Reddening.

   d. Decreased viscosity.

3. Fermentation of a carbohydrate in purple broth is indicated by a change in color from purple to:

   a. Red.

   b. Green.

   c. Brown.

   d. Yellow.

4. Which of the following organisms is catalase positive?

    a. Alpha hemolytic streptococci (other than pneumococci).

    b. Gamma hemolytic streptococci.

    c. Pneumococci.

    d. Staphylococci.

5. A rapid test to distinguish a suspected staphlococcal colony from streptococci is:

    a. Gelatin liquefaction.

    b. The catalase test.

    c. Mannitol fermentation.

    d. Growth on 7.5 percent sodium chloride.

6. The citrate test is based on an organism's ability to utilize citrate as the sole source of:

    a. Hydrogen.

    b. Iron.

    c. Nitrogen.

    d. Carbon.

7. Coagulase is an enzyme produced by pathogenic:

    a. Streptococci (other than pneumococci).

    b. Pneumococci.

    c. Organisms of all types.

    d. Staphylococci.

8. A positive coagulase test is indicated by:

    a. ALL OF THE BELOW.

    b. Clotting of plasma.

    c. Clearing of test solution.

    d. Change to a green color.

9. A positive ferric chloride test indicates the beta presence of group _____ hemolytic streptococci.

    a. A.

    b. B.

    c. C.

    d. D.

10. A positive ferric chloride test is revealed by the formation of a:

    a. Red color.

    b. Clear solution.

    c. Colorless solution.

    d. Permanent precipitate.

11. If the lead acetate paper test for hydrogen sulfide production is positive, the strip turns:

    a. Red.

    b. Green.

    c. Black.

    d. Yellow.

12. The lead acetate paper test is especially useful for differentiation of species of:

    a. Brucella.

    b. Streptococcus.

    c. Mycobacterium.

    d. Enterobacteriaceae.

13. A positive indole test is indicated by which color at the interphase between reagent and broth culture?

    a. Red.

    b. Blue.

    c. Green.

    d. Yellow.

14. A positive methyl red test indicates that an organism produces a:

    a. Low acid content from glucose.

    b. Low acid content from lactose.

    c. High acid content from glucose.

    d. High acid content from lactose.

15. A positive methyl red test is indicated by which color?

    a. Red.

    b Blue.

    c. Green.

    d. Yellow.

16. The nitrate reduction test utilizes which of the following?

    a. Ten percent ferric chloride.

    b. Sulfanilic acid and amyl alcohol.

    c. Potassium hydroxide and alpha-naphthylamine.

    d. Sulfanilic acid and N-N-dimethyl-I-naphthylamine.

17. Which of the following indicates a negative nitrate reduction test?

    a. Red color without the addition of zinc.

    b. Red color after the addition of zinc.

    c. No color after the addition of zinc.

18. In a typically positive oxidase test (alternate method), the colonies should finally:

    a. Clot.

    b. Dissolve.

    c. Turn pink.

    d. Turn black.

19. Unrease-positive organisms hydrolyze _____ to form the compound _____.

    a. Urea; ammonia.

    b. Acetamide; acetic acid.

    c. Urea; ammonium cyanate.

    d. Phenol red; carbon dioxide.

20. Which of the following is usually Voges-Proskauer-positive?

    a. E. coli.

    b. Shigella.

    c. Klebsiella.

    d. Salmonella.

21. A positive Voges-Proskauer test is indicated by what color?

    a. Blue.

    b. Green.

    c. Yellow.

    d. Pink or red.

22. On initial isolation, colonies of Staphylococcus aureus typically show a _____ pigmentation.

    a. White.

    b. Pinkish.

    c. Golden.

    d. Reddish.

23. When grown on blood agar, Staphylococcus aureus colonies usually cause:

    a. No hemolysis of the surrounding RBC's.

    b. An alpha type of hemolysis of the surrounding RBC's.

    c. A gamma type of hemolysis of the surrounding RBC's.

    d. Complete hemolysis of the surrounding RBC's.

24. Staphylococcus epidermidis is similar to Staphylococcus aureus in:

    a. Gram morphology.

    b. The ability to produce coagulase and ferment mannitol.

    c. The ability to produce toxins.

    d. The ability to hemolyze erythrocytes.

25. The typical morphological arrangement of the staphylococci is:

    a. Irregular clumps resembling grape-like clusters.

    b. Long chain formations.

    c. Pairs of slightly elongated cocci.

    d. Tetrads.

26. The primary means of distinguishing Staphylococcus aureus from Staphylococcus epidermidis is that Staphylococcus aureus:

    a. Ferments mannitol but does not produces coagulase.

    b. Hemolyzes RBC's producing an alpha hemolysis.

    c. Is nonhemolytic.

    d. Ferments mannitol and produces coagulase.

27. The streptococci are characterized as being:

    a. Nonmotile and nonsporeforming.

    b. Motile and sporeforming.

    c. Spore forming in broth cultures.

    d. Motile in broth cultures.

28. In the Lancefield classification of beta hemolytic streptococci, which group is most often associated with communicable diseases in humans?

a. A.

b. B.

c. C.

d. D.

29. The most useful method for preliminary differentiation of species of Streptococcus is:

a. Gram staining reaction.

b. Cellular morphology.

c. Size of colonies on blood agar.

d. Pattern of hemolysis produced on blood agar.

30. The true hemolytic reaction of a streptococcal strain may be altered by the incorporation of _____ in the agar.

a. Blood.

b. Glucose.

c. Peptone.

d. Ascitic fluid.

31. The formation of a green zone around certain strains of streptococci on blood agar usually indicates what type of hemolysis:

a. Alpha.

b. Beta.

c. Gamma.

d. Alpha prime.

32. A clear, colorless zone around a colony on blood agar usually indicates what type of hemolysis?

    a. Alpha.

    b. Beta.

    c. Gamma.

    d. Alpha prime.

33. Gamma forms of streptococci produce _____ on blood agar.

    a. A clear zone of hemolysis.

    b. A green zone of hemolysis.

    c. No hemolysis.

    d. A pink zone of hemolysis.

34. Beta hemolytic streptococci that are susceptible to bacitracin probably belong to group:

    a. A.

    b. B.

    c. C.

    d. D.

35. Beta hemolytic streptococci that are sodium-hippurate-positive probably belong to group:

    a. A.

    b. B.

    c. C.

    d. D.

36. Beta hemolytic streptococci that are bile-esculin-positive probably belong to group:

    a. A.

    b. B.

    c. C.

    d. D.

37. Beta hemolytic streptococci that are bacitracin-resistant, sodium-hippurate negative, and bile-esculin-negative probably belong to group:

    a. A.

    b. B.

    c. C.

    d. D.

38. Beta hemolytic streptococci that grow in an infusion broth with 6.5 percent NaCl probably belong to which of the following groups?

    a. A.

    b. B.

    c. C.

    d. D streptococci.

    e. D enterococci.

39. The pneumococci are slightly elongated gram-positive cocci characteristically arranged:

    a. Pairs.

    b. Clusters.

    c. Long chains.

    d. Packets of four.

40. Due to their hemolytic reaction, pneumococcal colonies growing on blood agar could very easily be mistaken for other:

    a. Beta hemolytic streptococci.

    b. Gamma hemolytic streptococci.

    c. Alpha hemolytic streptococci.

    d. Hemolytic staphylococci.

41. When people speak of "pneumonia" they are generally referring to lobar pneumonia. The most common cause of lobar pneumonia is:

    a. Stretococcus (Diplococcus) pneumoniae.

    b. Neisseria meningitidis.

    c. Mycobacterium tuberculosis.

    d. Corynebacterium diphtheriae.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES, LESSON 3

1. c. (paras 3-1a, b(1))
2. a (paras 3-1a, d(1))
3. d (para 3-2d)
4. d (para 3-3c(2))
5. b (para 3-3c(2))
6. d (para 3-4a)
7. d (para 3-5a)
8. b (para 3-5e(1))
9. b (para 3-6a)
10. d (para 3-6d)(1))
11. c (para 3-8d(1)
12. a (para 3-8d(3))
13. a (para 3-9c(2))
14. c (para 3-10a,d(1))
15. a (para 3-10d(1))
16. d (paras 3-11b(1), (2))
17. b (para 3-11d{2)(b))
18. d (para 3-12d(3))
19. a (para 3-13a)
20. c (para 3-14a)

21. d (para 3-14d(1))

22. c (para 3-17e)

23. d (para 3-17e)

24. a (para 3-18a)

25. a (para 3-18a)

26. d (para 3-19f; fig. 3-1)

27. a (para 3-20a)

28. a (para 3-20c)

29. d (para 3-21)

30. b ((para 3-21a)

31. a ((para 3-21c(1))

32. b (para 3-21c(2))

33. c (para 3-21c(3))

34. a (para 3-25a)

35. b (para 3-25b; table 3-1)

36. d (para 3-25d; table 3-1)

37. c (table 3-1)

38. e (para 3-25e)

39. a (para 3-27)

40. c (para 3-31c)

41. a (para 3-27; 3-30a)

End of Lesson 3

LESSON ASSIGNMENT

LESSON 4	Gram-Negative Cocci and Gram-Positive Bacilli.
LESSON ASSIGNMENT	Paragraphs 4-1 through 4-50.
LESSON OBJECTIVES	Upon completion of this lesson, you should be able to

4-1. Make correct paired associations among names of organisms, types of specimens, techniques of processing specimens, types of media, discriminating characteristics of colony morphology, microscopic morphology, biochemical reactions, pathogenicity, and interpretation of results for:

a. Pathogenic neisseriae.
b. Common corynebacteria.
c. Listeria monocytogenes.
d. Erysipelothrix rhusiopathiae.
e. Bacillus anthracis.
f. Common clostridia.
g. Pathogenic mycobacteria.

SUGGESTION After reading and studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 4

GRAM-NEGATIVE COCCI AND GRAM-POSITIVE BACILLI

Section I. INTRODUCTION

4-1. GENERAL COMMENTS ABOUT THE NEISSERIAE

The genus Neisseria consists of gram-negative cocci occurring predominantly in pairs. Neisseria gonorrhoeae is responsible for gonorrhea while Neisseria meningitidis is the cause of epidemic cerebrospinal meningitis. These human pathogens are usually found intracellularly within white blood cells. The nonpathogenic Neisseria species occur extacellularly and are important only because they may be mistaken for pathogenic forms. All the neisseriae may be encountered in the respiratory tract. Neisseria gonorrhoeae infections are usually venereal in origin, while Neisseria meningitidis infections are transmitted by asymptomatic "carriers" harboring meningococci in their nasopharynx.

4-2. COMMON CHARACTERISTICS OF THE NEISSERIAE

The neisseriae characteristically appear as gram-negative diplococci that are approximately 0.6 by 0.8 microns in size. The organisms do not form spores and are nonmotile. In stained smears of pus or body fluids the paired cells often have the shape of coffee beans or kidney beans, joined together on their concave or flattened sides. In pure culture, the characteristic reniform morphology is usually less apparent, and the majority of cells are oval or spherical and commonly aggregated in irregular masses. Cultures examined between 24 and 48 hours exhibit considerable variation in cellular size. In terms of gram morphology, the Neisseria species are all gram-negative diplococci, and there is no species differentiation on the basis of the gram morphology between the pathogens and the nonpathogens.

Section II. NEISSERIA GONORRHOEAE

NOTE: This section is adapted from materials prepared by the Center for Disease Control, Public health Service.

4-3. PATHEGENICITY

Gonorrhea, caused by Neisseria gonococci (gonococci), is the most prevalent venereal disease with over 42,000 cases per week estimated for the United States. The gonococci invade the mucous membranes resulting in acute inflammation and suppuration. In untreated cases such infections may extend to involve deeper tissues.

a. Typical gonorrhea of males is a urethritis characterized by the exudation of greenish-yellow pus and painful urination. In later stages of infection, the prostate and epididymis may become involved. Following regression of urethral discharge, the formation of fibrotic tissue sometimes leads to urethral stricture.

b. The gonorrhea of females, the infection usually spreads from the vagina to the urethra, cervix, and rectum. Such infections give rise to a mucopurulent discharge. In chronic infections, the fallopian tubes, ovaries, and peritoneum are involved with considerable frequency. The gonococci may invade the blood stream from localized infections. The areas of the body where lesions may be formed include the joints, heart valves and meninges.

c. Neisseria gonococci have been known to infect the eye of the newborn during passage through the birth canal of an infected mother. The conjunctivae are initially involved, but the infection rapidly spreads, if untreated, to all structures of the eye and usually results in permanent blindness. In the United States the incidence of gonorrheal conjunctivitis has been greatly decreased by the mandatory requirement that silver nitrate, penicillin ointment, or other suitable medications be instilled into the conjunctival sac of all the newborn.

4-4. CRITERIA FOR WOMEN

a. **Recommended.**

(1) To diagnose gonorrhea in women, specimens should be obtained from endocervical and anal canals and inoculated separately onto modified Thayer-Martin medium (MTM medium): Thayer-Martin medium with 2 percent agar, 0.25 percent dextrose and 5 mcg ml trimethoprim lactate) in culture plates, bottles or other suitable containers. In a screening situation, only culture specimens from the endocervical canal are recommended. The combination of a positive oxidase reaction of typical colonies containing typical gram-negative diplococci grown on this medium provides sufficient criteria for presumptive identification of Neiserria gonorrhoeae, (See below (para 4-6) "Special Situations" for criteria for confirmatory identification of isolates, especially those obtained from non-anogenital sites).

(2) Tests of cure are recommended for all women treated for gonorrhea. For test of cure, culture specimens should be obtained from both the endocervical and the anal canals, inoculated on MTM medium and interpreted as in (1).

(3) Oropharyngeal specimens (inoculated on MTM medium) should be obtained from all patients suspected of having disseminated gonococcal infection or pharyngeal gonococcal infection. Once pharyngeal gonococcal infection has been demonstrated, at least two pharyngeal specimens should be obtained after treatment in order to document cure (see below "Special Situations" para 4-6).

b. **Not Recommended.**

(1) Gram-stained or fluorescent antibody-stained smears are not recommended for the diagnosis of gonorrhea in women except as an adjunct to the cultures. Although gram-stained smears from the endocervical canal may be quite specific if examined by well-trained personnel, they are not adequately sensitive to rule out gonorrhea.

(2) Neither gram-stained nor fluorescent antibody-stained smears are recommended as a test of cure in women.

4-5. CRITERIA FOR MEN

a. **Recommended.**

(1) Microscopic demonstration of typical gram-negative, intracellular diplococci on smear of a urethral exudate constitutes sufficient basis for a diagnosis of gonorrhea. Prepare the smear by rolling the swab on the slide. Do not rub the swab on the slide because microscopic morphology will be distorted.

(2) When gram-negative diplococci cannot be identified on direct smear of a urethral exudate, or when urethral exudate is absent, a culture specimen should be obtained from the anterior urethra and inoculated on MTM medium. The combination of a positive oxidase reaction of typical colonies containing typical gram-negative diplococci grown on this medium provides sufficient criteria for presumptive identification of Neisseria gonorrhoeae (para 4-6)

(3) Where homosexual contact is suspected, additional culture specimens should be obtained from the anal canal and oropharynx and should be inoculated on MTM (para 4-6).

(4) Tests of cure are recommended for all men treated for gonorrhea and all sites that were infected before therapy should be retested. This is accomplished by inoculating a culture specimen from these sites on MTM medium; cultures should be obtained and interpreted as in (2) above.

(5) Pharyngeal gonococcal infection--see paragraph 4-4a(3).

b. **Not Recommended.**

(1) Fluorescent antibody stain of smears of urethral exudates is not recommended in men.

(2) A negative gram stain of urethral exudates should not be accepted as evidence of cure.

4-6. SPECIAL SITUATIONS

a. Sugar fermentation or fluorescent antibody reactions should be used to confirm presumptive identification of Neisseria gonorrhoeae in all cases of isolates obtained from other than anal or genital sites. In addition, sugar fermentation of fluorescent antibody reaction should be used for specific identification of organisms isolated on MTM medium from the anogenital sites in situations where gonococcal infection appears unlikely (e.g., in low-prevalence populations), and in special social, medicolegal and research situations.

b. Culture of blood or synovial fluid on enriched broth medium (such as Trypticase soy broth supplemented with 1 percent Isovitalex, 10 percent horse serum and 1 percent glucose) is a recommended procedure in special situations such as suspected gonococcal arthritis or septicemia. Specimens from conjunctive should be inoculated on MTM medium and chocolate agar supplemented with 1 percent Isovitalex. Identification of Neisseria gonorrhoeae should include sugar fermentation or fluorescent antibody techniques.

c. Gram-staining and specific fluorescent antibody staining of smears from conjunctivae, joint fluids, or skin lesions can be used as an adjunct in the diagnosis of gonococcal infections of these sites, particularly when partial therapy may prevent cultural recovery of organisms.

4-7. OBTAINING SPECIMENS FOR CULTURE--WOMEN

These procedures are provided for comprehensiveness only. They are usually performed by a nurse or physician.

a. **Endocervial Canal.** This is the best site to culture in women. Clinic personnel should:

(1) Moisten speculum with warm water; do not use any other lubricant.

(2) Remove excessive cervical mucus; do not use nay other lubricant.

(3) Insert sterile cotton-tipped swab into endocervical canal; move swab from side to side; allow 10 to 30 seconds for absorption of organisms onto the swab.

b. **Anal Canal (Also Called "Rectal Culture").** This specimen can easily be obtained without using an anoscope. Clinic personnel should:

(1) Insert sterile cotton-tipped swab approximately one inch into the anal canal.

(2) Move swab from side to side in the anal canal to sample crypts: allow 10 to 30 seconds for absorption of organisms onto the swab.

c. **Urethra or Vagina.** Cultures are indicated when the endorcervical culture is not possible; e.g.; hysterectomy patients and children.

(1) Urethra. Clinic personnel should:

(a) Strip the urethra toward the orifice to express exudate.

(b) Use sterile loop or cotton swab to obtain specimen.

(2) Vagina. Clinic personnel should use a speculum to obtain specimen from the posterior vaginal vault or obtain specimen from the vaginal orifice if the hymen is intact.

d. **Orapharynx.** This is common local source for disseminated gonococcal infection. Swab the poster for pharynx and tonsillar crypts with a cotton-tipped applicator.

4-8. OBTAINING SPECIMENS FOR CULTURE--MEN

a. **Urethra.**

(1) A culture is indicated when the gram stain of urethral exudate is not positive, in tests of cure, or as a test for asymptomatic urethral infection.

(2) Use sterile bacteriologic wire loop to obtain specimen from anterior urethra by gently scraping the mucosa. An alternative to the loop is a sterile calcium alginate urethral swab that is easily inserted into the urethra.

b. **Anal Canal.** This culture can be taken in the same manner as for women.

c. **Oropharynx.** This culture can be taken in the same manner as women

4-9. CONDITIONS FOR INOCULATION OF MODIFIED THAYER-MARTIN MEDIUM

Modified Thayer-Martin medium is selective for pathogenic neisseriae.

a. Medium should be at room temperature before inoculation.

b. Do not place inoculated culture medium in the refrigerator or expose it to extreme temperatures.

c. MTM medium in plates is the medium of choice. Bottled MTM ("Transgrow") a selective medium for the transport and cultivation of N. gonorrhoeae is recommended only when specimens cannot be delivered to the laboratory or incubator on the day they are taken. Validity of culture results depends on proper techniques for obtaining, inoculating, and handling specimens.

d. The storage life of MTM medium in plates, not sealed to prevent drying, and stored at room temperature, is only two weeks. MTM medium in plates sealed in plastic and refrigerated has a shelf life of 4 to 6 weeks. MTM medium in bottles, when refrigerated, has a shelf-life of 3 months. All media should be stored according to the directions supplied by the manufacturer.

4-10. MODIFIED THAYER-MARTIN MEDIUM IN PLATES

a. Roll swab rectangular plate in a large "Z" pattern on MTM medium in a round or rectangular plate.

b. Cross-streak immediately with a sterile wire loop or the tip of the swab in the clinical facility. (If cross streaking has inadvertently been omitted in the clinical facility, it should be done in the laboratory before incubation).

c. Place culture in CO_2-enriched atmosphere (e.g., candle jar) within 15 minutes. (Be sure to relight the candle each time the jar is opened.) Deliver to the laboratory as soon as possible.

d. Begin incubation of plates within a few hours (the sooner the better) at 35º to 36º C.

4-11. CO_2-GENERATING TABLETS

Several systems now use a CO_2-generating tablet to create CO_2 enriched atmosphere in an enclosed container (for example, special plastic bag). Care must be taken that:

a. The tablet must be dry and used before the expiration date.

b. The tablet must be placed within the chamber immediately after the medium is inoculated.

c. The medium be sufficiently moist to create the humid atmosphere necessary for release of CO_2.

d. The chamber be tightly sealed or closed before incubation.

4-12. MODIFIED THAYER-MARTIN MEDIUM BOTTLES

These include a 10 percent atmosphere (previously called "Transgrow").

a. Inoculate specimens on the surface of medium as follows:

CAUTION: Keep neck of bottle in upright position to prevent $C0_2$ loss.

(1) Remove cap of bottle only when ready to inoculate medium.

(2) Soak up all excess moisture in bottle with specimen swab and then roll swab from side to side across the medium, starting at the bottom of the bottle.

(3) Tightly cap the bottle immediately to prevent loss of CO_2.

b. When possible, incubate the bottle in an upright position at 35º to 36º C for 16 to 18 hours before sending to the laboratory, and note this on the accompanying request form. Resultant growth usually survives prolonged transport and is ready for identification upon arrival at the laboratory. (If an incubator is not available, store culture at room temperature (25º C or above) for 16 to 18 hours before subjecting it to prolonged transport and/or extreme temperatures.)

c. Package the incubated culture bottle and request form in a suitable container to prevent breakage, and immediately send it to a central bacteriologic laboratory by postal service or other convenient means.

d. At the laboratory, preincubated bottles will be examined immediately for Neisseria gonorrheae; other bottles will be incubated at 35º to 36º C for 24 to 48 hours and examined.

4-13. INCUBATION

a. Incubate all cultures not having growth.

(1) Adjust the incubator at 35º to 36º C since some strains of gonococci do not grow well at 37.5º C or above.

(2) CO_2 incubators should be maintained at 50-70 percent humidity and 5-10 percent CO_2 concentration; check CO_2 concentration at least once a day.

(3) A candle jar, or plastic bag with CO_2-generating tablet, providing CO_2 -enriched atmosphere should be airtight. (In a candle jar use a short, thick, smokeless candle fixed to a slide. The burning candle will generate approximately three percent CO_2 before extinction.)

(4) Incubate MTM in bottles in an upright position. (If the bottle cap is loose when received, incubate loosely capped bottle in CO_2-enriched atmosphere.)

b. After 20 to 24 hours incubation, examine plates and bottles for growth; return cultures without growth to the incubator for a total of 42 to 48 hours incubation.

4-14. PRESUMPTIVE IDENTIFICATION OF NEISSERIA GONORRHOEAE

a. Examine incubated MTM plates and bottles suspected for colonies suspected to be gonococci, using a high-intensity desk lamp.

(1) On primary isolation on MTM plates, gonococcal colonies appear glistening, grayish-white, raised, finely granular, moderately convex, and may vary in size. These are usually mucoid after 48 hours incubation. Colony size may depend on the age and surface moisture of the medium and the crowding on the plate.

(2) On MTM bottles, colonies may not have the typical appearance described above.

b. Perform oxidase test (Lesson 3 (para 3-12) on suspicious colonies.

(1) The members of genus Neisseria, as well as several other bacterial species, are oxidase positive.

(2) The color changes (pink to dark red to black) produced by the oxidase (dimethyl) reagent in contact with the colony are readily observed.

(3) The oxidase reagent is toxic for the gonococcus and required subcultures must be made as soon as a color change is apparent.

(4) If no characteristic colonies are seen after 42 to 48 hours incubation, flood the surface of the medium with oxidase (dimethyl) reagent to detect oxidase-positive microcolonies before discarding the culture as negative.

c. Prepare a thin smear of an oxidase-positive colony in a drop of water on a slide; air dry, heat-fix until warm to the back of the hand, and gram stain. Examine microscopically at 950-1000X magnification for typical gram-negative diplococci.

d. For routine anogenital specimens cultured on selective MTM medium, the combination of (1) typical colonies, (2) a positive oxidase reaction, and (3) typical gram-negative diplococcal morphology provides sufficient criteria for a presumptive identification of N. gonorrhoeae.

REPORT: "Presumptive identification Neisseria gonorrhoeae"

OR

"Oxidase positive, gram-negative diplococci morphologically compatible with Neisseria gonorrhoeae isolated."

CAUTION: Isolates from a specimen taken from other than an anogenital site that are presumptively identified as N. gonorrhoeae should be confirmed as N. gonorrhoeae by carbohydrate utilization tests or by fluorescent antibody staining before issuing a report.

e. Report negative cultures: Neisseria gonorrhoeae not isolated.

f. Report cultures overgrown with contaminating organisms: "Unsatisfactory-overgrown."

4-15. CONFIRMATORY IDENTIFICATION OF N. GONORRHOEAE

Local policy and special situations will determine the necessity for confirmatory identification of N. gonorrhoeae by carbohydrate utilization reactions or fluorescent antibody staining.

4-16. ARBOHYDRATE UTILIZATION TESTS FOR NEISSERIA GONORRHOEAE

a. A presumptive identification (table 4-1) of a culture as Neisseria gonorrhoeae may be confirmed by carbohydrate utilization patterns. N. gonorrhoeae produces acid (no gas) in glucose only.

b. The medium that the carbohydrates are added must be free of sugars and must readily support the growth of freshly isolated gonococci. Cystine Trypticase agar 9CTA), or equivalent, containing phenol red as an indicator of acid production may be used as a basic medium for carbohydrates utilization tests. As acid is produced (positive reaction), the medium changes from red to yellow. While this medium will support growth of practically all gonococci, some stains either do not grow or grow poorly. If sterile serum enrichment is added to enhance growth, it should first be inactivated at 56º C for 30 minutes. Calf or rabbit serum (5 percent) is more suitable than sheep or horse serum because of the latter's strong maltase activity.

c. CTA-carbohydrate media may be prepared as follows:

(1) Add 150 ml of distilled water to 4.3 grams of the dehydrated CTA medium; mix thoroughly, and heat with frequent agitation.

(2) Cool to 56º C and adjust the pH to 7.6 with 0.1 or 1.0N Na0H, and dispense in 50-ml amounts to 250-ml Erlenmeyer flasks. Stopper the flasks with cotton and sterilize at 15 pounds (121º C) for 15 minutes.

(3) Prepare 20 percent solutions of glucose, maltose, and sucrose in distilled water, and dispense in test tubes. It is preferable to sterilize these by Seitz or other types of filtration. However, if the autoclave is used, avoid prolonging the sterilization period and overheating. When identification of other species is to be made (as for N. meningitidis, N. lactamica, sicca etc.), lactose and fructose media must also be prepared.

		ACID PRODUCTION FROM CARBOHYDRATE IN CTA BASE					GROWTH		
							MTM	Nutrient agar[6]	
Organism	Oxidase	Glucose	Maltose	Sucrose	Lactose (or ONPG)[7]	Fructose	35-36°C	35-36°C	22-25°C
N. gonorrhoeae	+	+	–	–	–	–	–	–	–
N. meningitides	+	+	+	–	–	–	+	– (+)	–
N. lactamica	+	+	+	–	+[3]	–	+	+ (–)	– (+)
N. sicca	+	+	+	+	–	+	–	+	+
N. mucosa[1]	+	+	+	+	–	+	–	+	+
N. subflava[2]	+	+	+	–[4]	–	–[4..5]	–	+ (–)	+ (–)
N. flavescens[2]	+	–	–	–	–	–	–	+	+
Branhamella (Neisseria) catarrhalis	+	–	–	–	–	–	– (+)	+ (–)	+ (–)
Moraxella osloensis (Mimeae polymorpha var, oxidans	+	–	–	–	–		– (+)		

1. Reduces nitrate to gas.
2. Yellow pigmentation on Loeffler's serum medium.
3. Most stains will produce acid in 48 hours in CTA-lactose.
4. Strains formerly classified as N. perflava are positive.
5. Strains formerly classified as N. flava are positive.
6. NaCl-free nutrient agar inoculated with a single drop of a broth culture.
7. Ortho-nitrophenyl_B-galactoside.

Table 4-1. Identification of Neisseria species.

(4) Add 2.5 ml of a sterile 20 percent carbohydrate solution to 50 ml of the cooled CTA medium (final concentration of 1 percent carbohydrate).

(5) Mix the carbohydrate in the medium and dispense in 2-ml amounts to sterile screw-capped 13x100-mm tubes. Store at 4º to 10º C.

d. The CTA media should be inoculated as follows:

(1) In order to have a more uniform inoculum in all carbohydrate tubes, prepare a heavy, turbid suspension of organisms from a purification plate in 9.5 ml of sterile trypticase soy broth (TSB) without dextrose, or equivalent, or sterile distilled water.

(2) Using a sterile plugged disposable capillary pipet, add 2 to 3 drops of the heavy suspension to the surface of the medium; mix into the medium just below the surface with a sterile loop or needle.

(3) Replace cap and tighten; incubate at 35º C without added CO_2.

(4) Examine tubes at 24, 48, and 72 hours for growth and production of acid (indicator changes from red to yellow).

(5) For tubes giving acid reaction, determine purity of culture by examining a gram-stained smear of the growth.

4-17. CONFIRMATION OF NEISSERIA GONORRHOEAE

If fluorescent antibody confirmation of a culture is required, prepare a thin smear for staining on a slide having a 6 mm diameter etched circle. Place a loopful of distilled water in the area of the circle. Lightly touch a loop or needle to the suspected colony. Emulsify the material in the water and spread as thinly as possible over the etched circle and the surrounding area or prepare in 0.5 ml of sterile distilled water a slightly opalescent suspension of the suspected colony and spread a small loopful over the etched circle. Air-dry the smear. (Smears may be prepared from a colony and stained with fluorescent labeled conjugate up to 15 minutes after the colony has been treated with oxidase reagent.)

a. The fluorescent antibody staining technique is described below.

(1) Thoroughly air-dry the smear.

(2) Place a full 3-mm loopful of anti-Neisseria gonorrhoeae conjugate evenly over the specimen within the 6-mm circle and incubate at room temperature for 5 minutes in a moisture chamber to prevent drying.

(3) Gently rinse smears in running distilled water. Air-dry or blot gently with clear bibulous paper.

(4) Mount with cover slip, using a mounting medium of nine parts glycerine and one part carbonate-bicarbonate buffer, pH 9.0.

(5) Examine the smears with a fluorescence microscope fitted with a 10X ocular and 100X oil immersion objective; a BG-12 primary filter, and a Corning 3-72 (3387) or equivalent secondary filter (GG9).

(6) The gonococci appear as yellow-green diplococci.

b. Use a positive urethral smear or smear prepared from a known fresh isolate of gonococci as a positive staining control. Use a smear of Neiserria meningitidis as a negative staining control, and a smear of boiled suspension of E. cloacae as a nonspecific staining control. (Smears may be stored in the freezer for use as controls.)

c. When purchasing conjugate, specify those "Tested by the Center for Disease Control (CDC) and found to meet CDC specifications." Each new lot of conjugate should be evaluated before use with known fresh isolates of N. gonorrhoeae and fresh isolates of N. meningitidis and with smears of a boiled suspension of E. cloacae.

Section III. NEISSERIA MENINGITIDIS

4-18. PATHOGENICITY

The portal of entry for meningococci (Neisseria meningitidis) is the nasopharynx. The organisms constitute part of the transient flora in immune individuals, producing no symptoms, or they may set up a local nasopharynx infection in the nonimmune. The infection may extend to the blood stream causing meningococcemia, which is characterized by high fever, hemorrhagic rash, and fulminating sepis. From the blood stream, the organisms generally spread to the meninges causing meningitis. Acute meningococcal meningitis begins very suddenly with a severe headache, stiff neck, and vomiting. Affected individuals may lapse into a coma within a few hours. Neisseria meningitidis has acquired an infamous reputation as a cause of epidemics of meningitis in adults at various military basic training centers. Some deaths usually accompany these epidemics. In many cases, the serotype of meningococcus responsible for these epidemics is resistant to penicillin and sulfa drugs. It has been estimated that the carrier rate during nonepidemic periods ranges from 5 to 30 percent; during epidemics the carrier rate may reach 80 percent of the population. If the organism reaches the meninges of the spinal column, spinal meningitis results.

4-19. SIMILARITY TO GONOCOCCI

The morphology and staining reactions of meningococci are similar to gonococci. Meningococci are paired gram-negative cocci with a coffee-bean appearance. They may be either extracellular or intracellular (phagocytized by leukocytes). Like gonococci, they are oxidase-positive. They are fastidious and require an enriched medium, such as blood agar, Mueller-Hinton agar, chocolate agar or modified Thayer-Martin medium. The colonies of meningococci are round, smooth, and practically nonpigmented. Cultivation in a 3 percent to 10 percent carbon dioxide atmosphere greatly aids in the growth of meningococci.

4-20. SOURCE

Meningococci may be isolated from spinal fluid, blood, material from petechial skin lesions, nasopharyngeal swabs, joint fluid, and pus.

4-21. IDENTIFICATION

a. An organism is assumed to belong to the genus Neisseria if it is oxidase positive and if it exhibits typical gram-negative diplococci.

b. In many cases laboratory confirmation of species is required. This may be accomplished by a careful study of growth characteristics and the reactions obtained in appropriate carbohydrate media as shown in Table 4-1. To biochemically identify all of the species of the genus, five carbohydrates are normally used. However, to establish species identification of the pathogens, three of these carbohydrates are usually employed: glucose, maltose, and sucrose. N. gonorrhoeae produces acid from glucose and is maltose negative and sucrose negative. Neisseria meningitidis on the other hand, is glucose positive, maltose positive, and sucrose negative. It should also be pointed out that Neisseria meningitidis can be further classified into serological types using specific antisera. Serotyping is usually done to support epidemiological studies.

Section IV. GRAM-POSITIVE BACILLI: CORYNEBACTERIA AND RELATED SPECIES

4-22. GENERAL COMMENTS ABOUT THE GRAM-POSITIVE BACILLI

Members of the genera Corynebacterium, Bacillus, Clostridium, and Mycobacterium are gram-positive bacilli.

a. Corynebacterium diphtheriae biotypes gravis, intermedius, and mitis cause the disease diphtheria. The genus Corynebacterium also includes saprophytic species (called diphtheroids) that are normal inhabitants of the respiratory tract. These require differentiation from C. diphtheriae when possible diphtheria exists.

b. The majority of Bacillus species are saprophytes found in soil, water, air, and on vegetation. The one pathogenic member is B. anthracis that causes anthrax.

c. Members of the genus Clostridium are strict anaerobes. The Clostridium species cause several diseases that include botulism, tetanus ("lockjaw"), and gas gangrene. The clostridia are commonly found in soil, especially manured soil, and are prevalent in the intestinal tracts of man and animals.

d. Acid-fast bacilli make up the genus Mycobacterium. Unlike most bacteria, these organisms stain with difficulty. They require either prolonged contact with the dye or the accompanying application of heat or surface-wetting agents to facilitate dye penetration of the cells. Once stained, the mycobacteria resist decolorization with acid-alcohol thus, they are designated "acid-fact bacilli."

4-23. GENERAL COMMENTS ABOUT THE GENUS CORYNEBACTERIUM

a. The corynebacteria are slender, gram-positive rods, usually aerobic, measuring from 1 to 6 microns in length and 0.3 to 0.8 microns in breadth. These bacilli usually exhibit considerable pleomorphism. In addition to occurring as straight or slightly curved rods, they are frequently observed to be swollen on one or both ends, resulting in club or dumbbell-shaped forms. The diversity of shapes is due to the irregular distribution of cytoplasmic granules (metachromatic, or Babes-Ernst, granules) that build up during growth and distort the cell wall. In stained smears, the metachromatic granules appear as deeply stained bodies against lighter areas of cytoplasm. This gives the cell a transverse-banded, barred, or beaded appearance. Metachromatic granulation is satisfactorily demonstrated using methylene in blue stain. The corynebacteria are characteristically arranged in palisades. V-or Y-shaped branching forms may also occur. Microscopic arrangements have been compared to Chinese letters composed with matches.

b. It is very important to remember that the saprophytic diphtheroids may resemble Corynebacterium diphtheriae. However, diphtheroids are usually short, thick, uniformly stained rods in palisade arrangement. In most cases these forms exhibit little or not pleomorphism.

c. The corynebacteria are nonmotile, nonsporogeneous, nonencapsulated, and stain gram-positive indicating they have retained the primary stain, crystal violet.

4-24. CULTIVATION OF CORYNEBACTERIA

a. Good growth of the corynebacteria (table 4-2) is usually obtained on enriched media such as blood agar; however, slants of Loeffler's serum medium, and plates of blood or chocolate agar to which potassium tellurite has been added, are recommended for primary isolation of diphtheria bacilli. The organisms are aerobic and develop well at 37º C. Under these conditions typical growth of Corynebacterium diphtheriae is usually formed after 18 to 24 hours of incubation on Loeffler's serum medium. On potassium tellurite agar, characteristic growth of isolates usually requires 48 hours of incubation. The tellurite salts will also reduce the number of contaminants that are usually present from a throat culture, especially the gram-negative bacteria.

b. The saprophytic Corynebacterium species (diphtheroids) of the respiratory tract generally produce more abundant growth on blood, Loeffler's and tellurite agars than do diphtheria bacilli. However, colony size is not necessarily a criterion for differentiating between pathogenic and saprophytic forms.

Species	**Blood Agar 18-24 Hours**	**Loeffler's Serum Medium 18-24 Hours**	**Potasium Tellurite Agar 48 Hours**
C. Diphtheriae, var. gravis	Small, gray, dull opaque colonies which are non-hemolytic.	Circular, convex, cream-colored colonies with raised centers.	Flat, irregular, slate-gray colonies with a dull surface (2-3 mm in diameter).
C. Diphtheriae, var. intermedius	Small, gray, dull opaque colonies which are non-hemolytic.	Similar to var. gravis	Circular, convex, colonies with brownish-gray color against a white background (0.2-0.3 mm, diameter).
C. Diphtheriae, var. mitus	Small, gray, dull opaque colonies which are usually hemolytic.	Similar to var. gravis	Black, convex, colonies with a glistening surface (1.0-1.5 mm, in diameter).
Diphtheroid bacilli	Colonies considerably variable which are non-hemolytic.	Similar to var. gravis	Flat colonies, light gray or dark in center with white or gray translucent periphery.

Table 4-2 Colony characteristics of corynebacteria.

4-25. PATHOGENICITY OF CORYNEBACTERIA

a. **Varieties.** Of the corynebacteria, only the three biotypes of Corynebacterium diphtheriae (gravis, mitis, and intermedius) are generally recognized as pathogens. The rare species C. ulcerans is also pathogenic. The diphtheroids that normally inhabit the mucous membranes of the respiratory tract and the conjuctiva (e.g., C, hofmanii and C. xerosis) are not usually associated with the diseases of man. Corynebacterium diphtheriae is usually found in the respiratory tract of asymptomatic carriers or infected individuals. The organism is rarely isolated fro the skin or wounds. Diphtheria bacilli are spread by nasal or oral droplets from infected persons or by direct contact. Susceptible individuals are primarily within the 5 to 14 year age group.

b. **Toxic Effects.** The virulent bacilli enter by way of the mouth or nose, invade the mucous membranes of the upper respiratory tract, multiply rapidly, and begin to produce a powerful exotoxin. The toxin is absorbed by the mucous membrane, resulting in acute inflammatory response and destruction of the epithelium. The exudation of fibrin, red blood cells, and white blood cells into the affected area results in the formation of a gray, clotted film, or "pseudomembrane" often covering the tonsils, pharynx, or larynx. As the disease progresses, the toxin is extended to more distant tissues causing necrosis, functional impairment, and sometimes gross hemorrhage of the heart, liver, kidneys, and adrenals. Neurotoxic manifestations are also evidenced by paralysis of the soft palate, eye muscles, or extremities. Diphtheria bacilli remain localized in the upper respiratory tract. It is the exotoxin, disseminated to the blood and deeper tissues, which accounts for the symptoms of systemic involvement. The potency of toxin excreted by a given variety of Corynebacterium diphtheriae determines the severity of the disease. Individuals possessing sufficient levels of specific neutralizing antitoxin in their blood stream and tissues are resistant to diphtheria. Susceptible individuals lack antitoxin immunity. Since the toxins produced by all three types of diphtheria bacilli are antigenically identical, infections or toxoid inoculations with anyone will impart immunity to all. Susceptibility or immunity to diphtheria can be determined by the Schick test.

4-26. LABORATORY IDENTIFICATION OF CORYNEBACTERIA

a. **General Identification.** Proper specimens must be collected and initially examined directly. Final identification can only be accomplished by careful studies of cultures and demonstration of the exotoxin production.

(1) Methylene blue stains of smears from swab materials should be rarely examined for the presence f diphtheria bacilli. In clinically typical diphtheria, lesions and pseudomembranes usually yield large numbers of the characteristic bacilli upon direct examination.

(2) Loeffler's serum slants and potassium tellurite agar plates previously inoculated with swab materials from possible diphtheria should be examined at the 24 to 48 hour intervals. Typical colonies on each of the media are given in Table 4-2 and should be verified as possible diphtheria bacilli by examining smears stained with methylene blue and Gram's stain. Corynebacterium diphtheriae usually grows more rapidly more rapidly and more luxuriantly on Loeffler's serum medium than do other organisms of the respiratory tract with the exception of the diphtheroids. The microscopic morphology of diphtheria bacilli is usually characteristic on Loeffler's medium, yet often lacking on tellurite agar. Although tellurite agar is inhibitory to many gram-positive cocci and particularly to gram-negative organisms, diphtheroids and staphylococci with grow on this medium.

(3) Corynebacterium diphtheriae can be identified with reasonable certainty by demonstrating production of toxin. Observation of typical colonial and microscopic morphology are merely presumptive. Carbohydrate fermentation studies and other biochemical tests are helpful. The toxigenicity of a given strain may be determined either in vivo or in vitro.

b. **Toxigenicity in Vivo.** The in vivo test employs two guinea pigs. The guinea pig is indispensable as a laboratory animal when attempting to identify diphtheria. It is the animal of choice for standardization of both toxin and antitoxin. It is also of value in establishing the virulence of a particular strain as well as identifying an atypical strain of diphtheria. The procedure for inoculation of the guinea pig should be followed very closely. Emulsify the growth of a Loeffler's slant in 3 to 5 ml of broth. Inject 0.1 to 0.2 ml intracutaneously on the shaved side of each of two guinea pigs. One pig will serve as a control animal. The animal is given a protective dose of 500 units of antitoxin intraperitoneally 12 to 24 hours prior to being inoculated with a possible virulent strain of diphtheria. The other guinea pig that is the test animal is given 30 to 50 units of antitoxin 3 to 4 hours after the injection with the broth culture to prevent premature death without interfering with the specificity of the skin test. The interpretation of the results is very simple. The animal that received the large dose of antitoxin should show no reaction at the site of injection. The test animal that received the small dose of antitoxin should show an inflamed area after 24 hours and will progress to necrosis by 48 to 72 hours. It is interesting to note that slow toxin producers are negative on the plate method but are positive in the guinea pig test. One caution is to be observed when employing the animal test. If Staphylococcus, Streptococcus, or other organisms are present in a mixed culture from the throat and they are sufficiently virulent, lesions will appear in both animals. As many as six different cultures can be tested simultaneously on two guinea pigs.

c. **Toxigenicity in Vitro**. The other method of demonstrating the toxin-producing strains of diphtheria is an in vitro test using a KL virulence plate. This method uses a specially prepared solid medium and filter paper impregnated with diphtheria antitoxin. The organism is streaked across the plate perpendicular to the antitoxin paper strip and incubated 24 hours to 37º C. If the organism in question produces exotoxin, it diffuses into the medium. At the region of optimum proportions, a thin line of precipitate forms. This appears as "cat-whiskers" as the precipitate is formed at a 45º angle from the streak. If the organism fails to produce this characteristic precipitate, it is an avirulent strain of diphtheria or a diphtheroid.

d. **Biochemical Tests**. See table 4-3 for biochemical tests that can be useful in differentiating species of Corynebacterium.

	GLUCOSE	MALTOSE	SUCROSE	STARCH	TREHALOSE	NITRATE	UREASE	CATALASE	TOXIGENIC
C. diphtheriae	+	+	-	- or +[a]	-	+ or -[b]	-	+	+
C. ulcerans	+	+	-	+	+	-	+	+	+
C. haemolyticum	+[c]	+	+ or -	-	-	-	-	-	-
C. hoffmanii	-	-	-	-	-	+ or -	+	+	-
C. xerosis	+	+	+	-	-	+	-	+	-

Table 4-3. Differentiating of Corynebacterium species.

4-27. LISTERIA MONOCYTOGENES

Listeria monocytogenes is a small, nonencapsulated, nonsporogenous, gram-positive bacillus. It is aerobic to facultatively anaerobic in its oxygen requirements. This organism is motile at 22º C and much less motile at 37º C. The effect of temperature on the motility of this bacterium can be the key differentiating procedure" in its identification. Blood agar, phenyl ethyl alcohol agar, and serum tellurite agar are the media of choice. Much time and patience may be required in the initial culture of this organism. Many isolates have been successfully obtained only after prolonged incubation at 4º C for periods of 30 days or more. However, L. monocytogenes is not fastidious in its requirements once it has been isolated. The colony on blood agar is less than 1 mm in diameter and is characterized by a weak zone of beta hemolysis that may often be seen only when the colony is removed from the medium surface. Gram-stained smears reveal typical palisade arrangements. Infection with this organism may be transmitted to man from animals from unpasteurized milk, infected meat, and direct contact. The disease in man is usually a type of meningitis but may also cause abortion or stillbirth in women.

4-28. ERYSIPELOTHRIX RHUSIOPATHIAE

Erysipelothrix rhusiopathiae (also known as Erysipelothrix insidiosa) is a nonsporogenous, nonencapsulated, nonmotile, thick, gram-positive bacillus. It is isolated from blood culture or from characteristic lesions, resulting from contact with infected fowl, fish, or cattle. The organism grows well on 10 percent sheep blood agar at 30º to 37º C and is usually alpha hemolytic. The translucent, glistening I-mm colonies may mislead the observer into suspecting an alpha streptococcus; however, the gram stain will reveal the characteristic square-shaped rods in long tangled chains. This organism is catalase negative, nonmotile, and nitrate negative. Glucose and lactose are fermented without gas production while salicin is not utilized at all. E. rhusiopathiae will produce H_2S when tested by the lead acetate strip method.

Section V. GRAM-POSITIVE BACILLI: BACILLUS SPECIES

4-29. GENERAL COMMENTS ABOUT THE GENUS BACILLUS

The aerobic spore-forming bacilli include only one important pathogenic species, Bacillus anthracis. In size, this is one of the largest pathogenic bacteria. Identification is based on cell morphology, staining, and cultural characteristics. Members of the genus Bacillus are large, gram-positive, spore-forming rods, usually occurring in chains. Individual cells range between 1 to 1.25 microns in width and 3 to 10 microns in length. The only encapsulated and nonmotile species is Bacillus anthracis; the many saprophytic forms, B. subtilis, B. cereus, B. megaterium are nonencapsulated and are usually actively motile. The encapsulated cells of B. anthracis are usually found in direct smears of clinical specimen, but are rarely observed in smears of cultural growth. Most Bacillus species appear as long, straight-sided rods with curved ends; the cells of B. anthracis often possess swollen, square, or concave ends, which give the chains a bamboo-like appearance.

4-30. BACILLUS SPORE FORMATION

In an unfavorable environment most Bacillus species, including B. anthracis, produce spores. Spores of B. anthracis are not observed in specimens from living tissue. Although the spores of Bacillus species cannot be stained by ordinary methods, their presence in gram-stained smears is evidenced by unstained areas within the cytoplasm of vegetative cells. The Wirtz-Conklin technique employing heat, or the surface- active agent Tergitol, will satisfactorily stain the vegetative bacillary cells and spores of Bacillus species.

CAUTION: Ordinary heat fixation in preparing slides for staining will not destroy all anthrax spore. Strict aseptic technique should be used; a bacteriological hood may be employed in work with anthrax and other highly dangerous organisms.

4-31. BACILLUS CULTURES

All Bacillus species, including Bacillus antracis, grow rapidly on simple basic media. The addition specials enrichments such as blood or carbohydrates does not substantially improve growth. Certain strains are strictly aerobic; others are facultative. Growth occurs over a wide range of temperature for most species, especially the saprophytic forms. The optimum incubation temperature for Bacillus anthracis is 37º C. Spores are abundantly formed at 32º to 35º C. On blood agar after 18 to 24 hours incubation, typical colonies of Bacillus anthracis are 2 to 3 mm in diameter, off-white to gray opaque, dull, with irregular edges and a rough ground-glass appearance. Since B. subtilis, B. Cereus, B. megaterium, and other saprophytic species may exhibit the same colony picture, they are often referred to as pseudoanthrax bacilli. Hemolysis is an important basis for differentiation. The colonies of anthrax bacilli non hemolytic or weakly hemolytic on blood agar, while pseudoanthrax forms are usually surrounded by a definite zone of hemolysis. When anthrax is suspected and hemolysis is not present, an unknown Bacillus species must be further studied to prove or disprove its pathogenicity.

4-32. PATHOGENICITY OF BACILLUS

a. **Origin of Infections.** We are concerned pathologically only with Bacillus anthracis. Although this genus contains many saprophytes, B. subtilis, B. megaterium, and B. cereus are most often encountered. From a medical standpoint, these organisms are only important in that their microscopic and colonial morphology is often indistinguishable from the anthrax bacillus. The saprophytic species frequently occur as laboratory contaminants. This necessitates distinction of such from B. anthracis where possible anthrax exists.

(1) Anthrax is primarily a disease of herbivorous animals. Infections of sheep and cattle are most common. Horses, swine, and other animals are occasionally infected. The soil of grazing regions becomes contaminated with anthrax spores from carcasses of dead animals, and other-animals become infected during grazing. Viable spores enter the intestinal tract or the buccal mucosa, where they germinate and multiply. The bacilli are disseminated via the lymphatics to the blood stream and deeper tissues, rapidly resulting in death of the animal.

(2) Infections of man are almost always of animal origin. The organisms may enter through the skin, through the respiratory tract, or through the intestinal mucosa. The incidence of anthrax is highest among butchers, herdsmen, wool handlers, tanners, and other occupational groups dealing with, infected animals or their products.

b. **Forms of Infection.**

(1) Cutaneous anthrax is anthrax infection and most often-infected tissue, hides, hairs, or papule that rapidly progresses in sequence to a vescicle, pustule, and ultimately to a hard necrotic ulcer. Such infections may spread to deeper tissues resulting in septicemia and widespread involvement of internal organs.

(2) Primary pulmonary anthrax originates from inhalation of spores disseminated into the air in the process of handling infected materials, especially animal fibers (wool or fleece). Infected individuals exhibit signs of pneumonia, which often progresses to fatal septicemia. Fortunately, pulmonary infections are not very common.

(3) Intestinal anthrax may result from ingestion of insufficiently cooked meat of infected animals or from ingestion of goods contaminated with spores. Infections of the intestinal tract are very rare in man, but are the most common form of the disease in animals.

c. **Virulence.** Bacillus antracis produces no soluble exotoxin or endotoxin.

(1) Virulence is apparently associated with the ability to form a capsule. The capsule is composed on polypeptide (protein complex of d (-) glumatic acid) material instead of a polysaccharide substance common to capsules of most other bacteria.

(2) While the vegetative cells of Bacillus species are no more resistant to deleterious influences than other bacteria, the spores are highly resistant. Anthrax spores have been known to survive for decades in soil. The spores ordinarily require boiling for at least 10 minutes to effect their destruction. Treatment of the spores with disinfectants usually requires prolonged exposure. For example, 0.1 percent mercuric chloride may not destroy anthrax spores even after 72 hours. Standard sterilization temperatures and periods of exposure successfully destroy all pathogenic bacterial spores. Though the spores of saprophytic species exhibit comparable or even greater resistance, their medical importance is negligible although in the clinical laboratory they must be differentiated from the pathogenic species.

4-33. LABORATORY IDENTIFICATION OF BACI-SPECIES

a. **General Identification.** The laboratory identification of members of this group is dependent upon proper collection of appropriate specimens, and their direct examination and subsequent culture and subculture on appropriate media. In gram-stained smears of exudate and blood, Bacillus anthracis appears as large gram-positive bacilli, usually in chains of 2 to 6 cells. The bacilli may be numerous in blood smears from generalized anthrax. The capsules of anthrax bacilli cannot be adequately observed in gram-stained preparations, yet their presence may be noted as imperfectly stained, granular halos with ragged edges. Stained with Wright's or Giemsa's stain, anthrax bacilli in films of exudates or blood and in tissue impressions appear bluish black and are surrounded by a clearly defined, pinkish capsular substance. Regardless of direct findings, cultural results should be obtained before reaching a final diagnosis. Blood agar plates previously streaked with materials from infected tissues yield abundant growth in 24 hours under aerobic conditions at 37º C (table 4-4). The typical opaque, flat, irregular, gray-white colonies exhibiting no hemolysis and a ground-glass appearance should be selected for study. A semi-solid motility medium is inoculated with a straight needle. The motility tubes are examined for diffusion after 24 to 48 hours incubation at 37º C. Bacillus anthracis strains are nonmotile, while saprophytic Bacillus species are usually motile.

Organism	**Colonies On Blood Agar**	**Hemolysis**	**Motility**	**Capsule**	**Mouse or Guinea Pig Virulence**
Virulent B, anthracis	Rough, dull, and irregular ("frosted glass").	-	-	+	+
A Virulent B, anthracis	Rough, dull, and irregular ("frosted glass") or smooth or mucoid.	-	-	-	-

Table 4-4. Differentiation of Bacillus anthracic from saprophytic (pseudoanthrax) Bacillus species.

b. **Use of Laboratory Animals.** Fresh specimens of blood, sputum, macerated tissue and exudates from lesions, or saline suspensions from 24-hour nutrient agar slant cultures may be inoculated into suitable animals. White mice or guinea pigs give the best results. The specimen is inoculated subcutaneously into a previously shaved area on the abdomen of the animal. Large doses are not necessary because less than one hundred organisms will usually kill mice or guinea pigs. After 36 hours, animals may begin to exhibit evidence of illness. At this time, doughty swellings are often present around the area of inoculation. If the inoculum contains large numbers of anthrax bacilli, death may occur in less than 48 hours. However, 3 to 4 days are ordinarily required. Laboratory animals dying from anthrax exude a yellow, gelatinous substance in the subdermal area near the point of injection. Often this exudates may extend over large areas of the abdominal subcutaneous connective tissue. The blood vessels of this tissue and the mesenteries exhibit marked congestion, and hemorrhages are often seen near the injection site. The spleen is greatly enlarged and much darker that normal. Although anthrax bacilli can usually be demonstrated in stained smears of blood or any of the tissues, examination of the spleen is preferable. This is accomplished by excising a portion of the spleen and gently rubbing its cut section over the surface of clean slides using a pair of forceps. Films prepared in this manner should be stained with both Wright's and Gram's stain.

Section VI. GRAM-POSITIVE BACILLI: CLOSTRIDIA

4-34. GENERAL COMMENTS ABOUT THE GENUS CLOSTRIDIUM

a. The members of the genus Clostridium are large anaerobic gram-positive rods of variable length and breadth, ranging from long filamentous forms to short plump bacilli. In an appropriate environment, individual bacilli of most species produce a single spherical or ovoid spore that may be located centrally, subterminally, or terminally within the vegetative cell. In most instances the spores appear as swollen bodies since they are generally wider than the diameter of the rods in which they develop. The shape and position of the spore, as well as the swelling of the vegetative cell are characteristics that may contribute to species identification as shown in table 4-5.

b. Spores of Clostridium species are not stained by ordinary methods: therefore, in gram- and methylene blue-stained smears, spores are evidenced as unstained areas within the dark stained cytoplasm of vegetative bacilli or as free hyaline bodies. The relatively impervious spore bodies may be effectively stained using the Wirtz-Conklin technique. CAUTION: Ordinarily heat fixation in preparing slide for staining may not destroy all spores. Strict aseptic technique should be used; a bacteriological hood may be useful in work with spore formers.

c. The majority of the clostridia are motile, but Clostridium perfringens, the species most frequently isolated from clinical materials, is nonmotile.

Species	Surface colonies anaerobic blood agar	Motility	Spores	Fermentation (deep agar)			Indole Production	Nitrate reduction	Proteolysis (cooked meat)
				Dextrose	Lactose	Sucrose			
Cl. tetani	Small (1 mm) flat, rhizoid or feathery surface; raised centers; single hemolytic zone.	+	Round, terminal, and swollen (drumstick).	–	–	–	–	–	Gas.
Cl. botulinum	Large (5–10 mm); glistening translucent edges with brown centers; single hemolytic zone.	+	Oval, subterminal and swollen.	+	–	–	–	–	Gas, digestion blackening.
Cl. perfringens	Large (2–5 mm), round, smooth, opaque, entire; double hemolytic zones.	–	Oval, central, nonswollen (rarely present).	+	+	+	–	+	Gas.
Cl. novyi	Small, delicate, flattened, transparent; blue gray and irregularly contoured; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	–	–	–	–	Gas.
Cl. septicum	Small, with branching or rhizoid margins and deep, opaque centers; single hemolytic zone.	+	Oval, subterminal, swollen.	+	+	–	–	+	Gas.
Cl. sporogenes	Small, transparent, with rhizoid or amoeboid margins and raised centers; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	–	–	–	–	Gas, digestion blackening.
Cl. chauvoei	Small, flat, round or leaflike; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	+	+	–	+	Gas.
Cl. histolyticum	Minute, round, translucent, with "dew drop" appearance; single hemolytic zone.	+	Oval, subterminal and swollen.	–	–	–	–	–	Digestion blackening.
Cl. bifermentans	Small, transparent, with rhizoid margins; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	–	–	+	–	Gas, digestion blackening.

Table 4-5. Morphological and biochemical differentiation of clostridium species.

4-35. GROWTH REQUIREMENT OF CLOSTRIDIA

The clostridia are obligate anaerobes. Growth may be obtained over a wide range of temperatures; however, 37° C is optimum for pathogenic species. Although variations in nutritive requirements throughout the clostridia do exist, they may be successfully isolated from specimens using blood agar and thioglycollate broth (the addition of 0.6 percent glucose in helpful as an added growth factor). Anaerobic conditions may be provided by incubating the inoculated blood agar plates in a Brewer anaerobic jar, or any several other methods for providing strict anaerobic conditions (see section on anaerobic methods in Lesson 2, Section II).

4-36. APPEARANCE OF CLOSTRIDIA

Stained smears of growth usually reveal spores, except when Cl. Perfringens is present. This species fails to sporulate on most media, especially those media containing carbohydrates. On blood agar, after 48 hours anaerobic incubation at 37º C, typical colonies of the various Clostridium species appear as described in Table 4-5. Most clostridia produce distinct beta hemolysis on blood agar. Clostridium perfringens however, may exhibit a "target" appearance or double zone of hemolysis. This is shown by a definite narrow 1 to 2 mm zone, immediately around the colonies, which is surrounded by a wide 4 to 5 mm zone of partial hemolysis. Although the microscopic and colonial morphology of certain clostridia may appear quite distinctive, final identification rests with the performance and interpretation of biochemical tests.

4-37. PATHOGENICITY OF CLOSTRIDIA

Since the natural habitat of the clostridia is worldwide in soil or in the intestinal tract of man and animals, pathogenic species are always present and may cause disease when the opportunity arises. The pathogens are those organisms responsible for botulism, tetanus, and gas gangrene.

a. **Tetanus.** Clostridium tetani causes tetanus. This disease results from the introduction of spores (from soil or feces) of the organism into puncture wounds, burns, surgical sutures, or other traumatic injuries. Spores of the organism germinate to form the vegetative bacilli that multiply and produce a powerful exotoxin at the expense of the necrotic tissue, created by vascular destruction. The exotoxin spreads through rapid absorption and acts particularly on the tissue of the spinal cord and peripheral motor nerve endings. Toxemia is first evidenced by muscle spasms near the site of infection with subsequent spasms of the jaw muscles (lockjaw). The intoxication progresses to the nerves of other voluntary muscles causing tonic spasms, convulsions and, ultimately, death. Prevention of disease rests with active immunization of the populace with toxoid, or passive immunization with antitoxin as soon as possible after injury.

b. **Botulism.**

(1) Clostridium botulinum is responsible for a fatal type of food poisoning, botulism. The disease is an intoxication rather than an infection in that outbreaks occur following ingestion of food in which Cl. Botulinum has grown and produced a highly potent exotoxin. Within the anaerobic environment of the foodstuff, the spores germinate to form vegetative bacilli, which in turn, produce toxin. Since the spores of Cl. Botulinum will withstand a temperature of 100º C for at least 3 to 5 hours, they present a definite hazard to home canning. Toxin-containing foods may appear spoiled and rancid; cans may be swollen due to gas formation by the organism. In some cases, the foodstuff may appear entirely innocuous. The toxin is destroyed by hearing the food at 100º C for 10 minutes. Outbreaks of botulism are rare in the United States because of the rigid regulation of commercial canning and good preservation. Most cases since 1910 have seen those associated with home preparation of foodstuff.

(2) There are five antigenic types of Cl. botulinum toxin, designated A, B, C, 0, and E. Type A toxin is one of the most poisonous substances known. After approximately 18 to 48 hours following the consumption of toxic food, neurotoxic manifestation is evidence in visual disturbances, inability to swallow, and speech difficulty. Progressive signs of bulbar paralysis are exhibited and these lead to fatal termination from respiratory failure or cardiac arrest. Since the various toxin types are highly antigenic, potent antitoxins may be obtained by injecting toxoids into animals. Polyvalent antitoxin (A through E) is administered intravenously to affected individuals.

c. **Gas Gangrene.**

(1) The Clostridium species most commonly associated with gas gangrene are C. perfringens, C. novyi, and C. septicum. Clostridium perfringens is the most frequent cause and is found either alone or mixed with other anaerobes.

(2) Gas gangrene often develops as a complication of severe traumatic injuries such as dirty, lacerated wounds, especially those accompanying compound fractures. In these and other injuries, the circulation to a local tissue area is often impaired or destroyed. The resulting necrotic tissue, void of oxygen and rich in nutrients, affords an ideal anaerobic environment in which the spores of gangrene organisms may germinate and multiply. The organisms actively metabolize tissue carbohydrates to acid and gas. The gangrenous process extends to other tissues primarily as a result of exotoxins excreted by pathogenic clostridia. The exotoxins include hyaluronidase, lecithinase, and collagenase.

(3) In addition, other enzymes may be present which exhibit hemolytic, necrotizing and lethal effects on tissues. Gas gangrene is usually a mixed infection composed of toxigenic and proteolytic clostridia and other aerobic and anaerobic, gram-positive organisms.

(4) The accessory organisms may contribute to the severity of infection. Without the prompt administration of antitoxin or amputation of necrotic tissue, patients die from toxemia. The antitoxin employed usually consists of pooled concentrated immune globulins against toxin of Cl. perfringens, Cl. Novyi, and Cl. Septicum.

4-38. LABARORATORY IDENTIFICATION OF CLOSTRIDIUM SPECIES

a. **Specimens.**

(1) Although bacteriological examination of swab specimens and tissues from contaminated wounds may yield Clostridium tetani, such materials are rarely submitted for tetanus. Diagnosis of the disease is dependent upon the clinical picture and history of injury.

(2) In cases of botulism, specimens from the patient are almost of no value, since the disease is an intoxication rather than an infection. Laboratory diagnosis is accomplished by demonstrating Clostridium botulinum toxin in leftover food. The organism may be isolated from the food and identified on the basis of biochemical tests and the demonstration of toxin production.

(3) From cases of gaseous gangrene, exudates from wounds may be collected with a sterile swab or aspirated with a sterile syringe and needle. Infections due to Clostridium species are readily recognized when typical gaseous gangrene occurs. Any necrotic or devitalized tissue subject to contamination may yield clostridia on bacteriological examination.

b. **Direct Examination.** Gram-stained smears of specimens may reveal the presence of large gram-positive rods with or without spores. Frequently, specimens from gangrenous lesions are contaminated with gram-negative rods and gram-positive cocci. The bacilli of gaseous gangrene cannot be distinguished/ morphologically from the saprophytic putrefactive anaerobes that may be associated with gangrene. For this reason, direct smears are only of presumptive value. Materials from gangrenous lesions must be cultured.

c. **Examination of Cultures.**

(1) If growth of large gram-positive bacilli is obtained in an anaerobic culture, a member of the genus Clostridium should be suspected, provided aerobic plates are negative. If only the thioglycollate broth should yield gram-positive organisms from specimens, a loopful of the medium should be streaked to each of the two blood agar. One plate is incubated aerobically, the other anaerobically. This is essential since thioglycollate broth will support the growth of gram-positive rods, both aerobic (possible Bacillus species) and anaerobic (possible Clostridium species).

(2) In addition to Clostridium species, gangrenous infections may contain coliform bacilli, Pseudomonas species, or members of the genus Proteus. Under such circumstances, the primary anaerobic blood agar plate may be overgrown with these organisms, thereby making the isolation of Clostiridium species difficult or impossible. This may be overcome by incubating the primary thioglycollate broth cultures containing the gram-positive rods suggestive of Clostridium species, for 48 to 72 hours. Over this period of time, only the gram-negative bacilli will greatly decrease in numbers, allowing isolation of clostridia in subculture.

d. **Isolation Clostridium from Mixed Cultures.** When spores of a possible Clostridium species are observed in a mixed thioglycollate broth culture, their resistance to heat may be used advantageously in obtaining isolation. One-tenth milliliter of the mixed culture is aseptically inoculated to a fresh tube of thioglycollate broth. The freshly inoculated broth is then heated in a water bath at 80º C for 15 to 30 minutes. This will destroy all vegetative growth of bacteria, but will not destroy the spores. The heated medium is then incubated for 24 to 48 hours, resulting in a pure culture of the Clostridium species. When isolated colonies are obtained on blood agar, the growth should be carefully picked and transferred to thioglycollate broth. Following incubation, this pure culture is used to carry out the confirmatory studies.

e. **Confirmatory Laboratory Tests.** Cultural isolates may be distinguished as belonging to the genus Clostridium on the basis of strict anaerobiasis, and microscopic and colonial morphology. Final identification of a Clostridium species is dependent upon the results of biochemical studies and/or the demonstration of exotoxin production (C. tetani and C. botulinum).

Section VII. GRAM-POSITIVE BACILLI: MYCOBACTERIA

4-39. GENERAL COMMENTS ABOUT THE GENUS MYCOBACTERIUM

The genus Mycobacterium, whose members are also called the "acid-fast bacilli," contains many species of saprophytic acid-fast bacilli, but M. tuberculosis, M. bovis, M. avium, and M. leprae are clearly definable pathogens. M. tuberculosis is the principal cause of human tuberculosis. M. bovis, is primarily responsible for tuberculosis of cattle, although infections of cattle are transmissible to man. M. avium is infectious for fowl. The leprosy bacillus Mycobacterium leprae is the causative agent of human leprosy. In contrast to the other pathogenic mycobacteria, M. leprae has not been routinely grown in vitro. Human leprosy is generally not transmissible to animals.

4-40. PATHOGENICITY

a. **Human Infections.** Mycobacterium tuberculosis causes about 90 percent of all mycobacterial infections. It is the principal cause of tuberculosis in man. Among communicable diseases, tuberculosis is the leading killer in the world today, although it is no longer the leading cause of death in countries where the standard of living is high.

b. **Routes of Infection.** Tubercle bacilli may enter the body by way of the respiratory or alimentary tracts, as well as the conjunctiva. The respiratory tract is the most frequent and important route of infection for man. Infected individuals in the process of sneezing, coughing, or expectorating produce an infectious aerosol of droplets and contaminated dust particles, which may be inhaled by susceptible individuals. Infections are also acquired from fomites (towels, drinking cups, doorknobs, etc.). The ingestion of unpasteurized milk or inadequately cooked meat of infected cattle is an important source of infection where bovine tuberculosis is not well controlled.

c. **Spread of Disease Within the Body.** Following initial infection, tubercle bacilli form primary and secondary lesions within the tissues. The organisms may then spread to various tissues via the lymphatic system, blood stream, or by direct extension. Blood stream invasion results in the bacilli's being transported throughout the body and thus giving rise to acute military or chronic disseminated tuberculosis. Practically any tissue of the body is subject to invasion by tubercle bacilli. However, more than 90 percent of the deaths from tuberculosis are due to the pulmonary type. Infections of the bone and joints, lymphnodes, spleen, liver, kidney, meninges, and gastrointestinal tract do occur, but with much less frequency. Disseminated infections are somewhat more prevalent in children. The lesions formed by pathogenic acid-fast bacilli are referred to as tubercles. Tubercles may either rupture and discharge their bacilli to produce further infection, or they may heal and permanently wall-off the bacilli by fibrosis or calcification.

d. **Acquired Resistance.** If man or lower animals survive the first infection with tubercle bacilli, they acquire some resistance to tuberculosis. Upon subsequent infections, the defense mechanisms of these subjects have an increased capacity to localize tubercle bacilli. Although antibodies are formed by the host against a variety of cellular antigens within the tubercle bacilli, such antibodies appear to be of little value in increasing resistance. The increased resistance to infection is largely attributed to the mononuclear wells that acquire a greater ability to ingest tubercle bacilli. Mononuclear cells develop this property in the course of primary infections. Nevertheless, antibody production in response to tuberculous infections is of value in the diagnosis of tuberculosis. This forms the basis of the tuberculin skin test. Individuals who have had no contact with tubercle bacilli exhibit no reaction to this skin test; however, the majority of normal adults are tuberculin-positive. In children, however, positive reactions are more suggestive of active infections. The tuberculin test is valuable as a screening test in examining children for possible tuberculosis.

4-41. GENERAL PROCEDURES

a. The specimens examined for diagnosis of tuberculosis may consist of a variety of materials depending upon clinical manifestations. Sputum, urine, and gastric washings are more often analyzed, but tissue specimens, lymph aspirations, and pleural, pericardial, spinal, or joint fluids may be submitted upon occasions.

b. All clinical and laboratory materials suspected of being contaminated with pathogenic mycobacteria or related species should be handled with strict aseptic technique. These organisms are highly resistant to adverse environmental conditions. When flaming the wire of the inoculating needle, spattering of the specimen must be avoided. The use of a bacteriological hood is highly desirable and should be used when extensive work with acid-fast organisms is undertaken.

c. Smears should be prepared of all clinical specimens from suspected tubercular patients and stained by either the Ziehl-Neelsen or Kinyoun technique. A positive acid-fast stain is the first prerequisite for identification of organisms as mycobacteria.

d. The final decision as to whether or not the organism is a pathogen is determined by careful cultural studies and perhaps animal virulence tests. Although tubercle bacilli may be demonstrated in direct smears, the detection is usually more satisfactorily accomplished when the specimen is concentrated prior to staining. Concentrated material should be used for culture inoculations and animal injection.

e. To prepare smears for microscopic examination, specimens of sputum may be concentrated by mixing with an equal volume of 5 percent sodium hypochlorite, centrifuging, decanting the supernatant, transferring the sediment with a cotton-tipped applicator stick to a slide, and air drying. Sodium hypochlorite is used only to prepare stained smears. Since it is bactericidal, it should not be used with specimens for culture or animal studies.

4-42. APPEARANCE OF MYCOBACTEIRA

a. In tissues and exudates from infection, tubercle bacilli appear as small, thin rods with rounded ends. They may be straight or slightly curved and range between 2 to 4 microns in length and 0.3 to 1.5 microns in thickness. In smears from cultures, longer filamentous forms are occasionally observed, as well as swollen or club-shaped cells. Tubercle bacilli occur singly, in small groups, or occasionally in clumps of indiscernible cells. The irregular grouping of these forms is sometimes suggestive of branching. All of the mycobacteria are nonmotile and nonsporogenous. Virulent forms appear to produce a capsular substance, especially when grown on a serum-enriched medium.

b. Mycobacteria stain rather poorly with the gram stain, although they are gram-positive. This is due to large amounts of lipids, fatty acids, and waxes within the cells that impede penetration of the dye. Staining difficulties are overcome when special methods are employed such as the Ziehl-Neelsen or Kinyoun stain. Tubercle bacilli often appear as irregularly stained forms, exhibiting a banded or beaded appearance.

c. The leprosy bacilli and the saprophytic acid-fast organisms closely resemble the tubercle bacilli with regard to morphological and staining characteristics and cannot be distinguished from them on the basis of morphology alone. Leprosy is usually differentiated from tuberculosis on a clinical basis.

4-43. TRATION FOR CULTIVATION

a. **Principle.** The mucolytic compound N-acetyl-L-cysteine is used to help digest and decontaminate specimens for cultivation of mycobacteria.to help digest mycobacteria.

b. **Reagents.**

(1) Digestant. Mix 50 ml of (4 percent) sodium hydroxide, 50 ml of 0,IN (2.94 percent) sodium citrate .2H_20, and 0.5 gram of N-acetyl-L- cysteine powder. The solution is self-sterilizing but should be used within 24 hours.

(2) M/15 phosphate buffer, pH 6.6. Make Solution A by dissolving 9.47 grams of sodium monohydrogen phosphate (anhydrous) in 1,000 ml of distilled water. Make Solution B by dissolving 9.08 grams of potassium dihydrogen phosphate in 1,000 ml of distilled water. Mix 625 ml of Solution A and 375 ml of Solution B. Adjust the pH to 6.6. Dispense in appropriate containers. Sterilize.

(3) 0.2 percent bovine albumin.

c. **Procedure.**

(1) Transfer 10 ml of the sputum specimen to sterile, aerosol-free plastic centrifuge tube with a screw cap. THIS SHOULD BE DONE UNDER A WELL-VENTILATED HOOD.

(2) Add an equal volume of digestant.

(3) Tighten screw caps and ix well in a Vortex mixer for not more than 30 seconds.

(4) Allow to stand for 15 minutes.

(5) Fill the tube with M/15 phosphate buffer within 1/2 inch of the top.

(6) Centrifuge at r near 2,000 gravities for 15 minutes.

(7) Decant the supernatant into a can containing phenolic disinfectant. Wipe the tip of the tube with 5 percent phenol.

(8) Unless the quantity of sediment is very small, use a sterile applicator stick or flamed loop to make a 1x2-cm smear on a new microscope slide for Ziehl-Neelsen staining. If the quantity is very small, delay making smears until after the next step.

(9) Use a pipet to add 1 ml of 2 percent bovine albumin to the remaining sedimentation. Shake gently by hand. Refrigerate until inoculation is possible.

(10) Add 10 drops of the serum-sediment mixture to 4.5 ml of sterile water to make a diluted mixture.

(11) Seed each of the mixtures (diluted and undiluted) onto two tubes of an egg base medium (such as Lowenstein-Jensen) and onto a 7H11 agar plate (7HI0 with pancreatic digest of casein).

(12) The remainder of the mixtures can be used for drug-susceptibility testing or refrigerated for later culture.

4-44. CULTIVATION OF MYCOBACTERIA

The tubercle bacilla are aerobic and grow best in a carbon dioxide incubator. The saprophytic species of Mycobacterium grow readily on ordinary laboratory media at room temperature. An incubation temperature at 37º C is optimum for Mycobacterium tuberculosis and M. bovis while M. avium prefers a temperature of 40º to 42º C. Growth of these organisms is much less rapid than most bacteria, usually requiring from 2 to 60 weeks.

a. A highly enriched medium is required for the cultivation of the pathogenic species of Mycobacterium. Although many media have been used to cultivate tubercle bacilli, the egg media Lowenstein-Jensen medium and Petragnani's medium are widely used. The principal growth-promoting constituents of these agar media are homogenized whole egg, glycerol asparagin, and potato starch. Malachite green is also incorporated in each medium to inhibit growth of organisms other than mycobacteria.

b. In addition to a bottled or tubed egg medium such as one of these, it is useful to employ simultaneously a clear agar plate medium for earlier information about the cultured organisms. Middlebrook 7H10 agar is transparent medium that is becoming very popular as a plate medium for this purpose. Medium 7H11, an adaptation, is recommended in the procedure above.

c. Cultures for tubercle bacilli are incubated at 37º C for at least 8 weeks before being discarded as negative.

4-45. VIRULENT STRAINS OF MYCOBACTERIA

See Table 4-6 for characteristics of the acid-fast bacilli. Cording (tight serpentine cords observed microscopically) exhibited by these bacteria when grown upon complex, organic media and upon 7H10 medium is indicative of virulent tubercle bacilli. Cording tends to be characteristic of virulent strains. Virulent strains are capable of binding the dye of neutral red salts in an alkaline aqueous media. Saprophytic and virulent types are not capable of binding the dye in such a solution. Acid-fast bacilli exhibit a slight endotoxin when injected into animals in large amounts. No apparent exotoxin are synthesized. Catalase is produced by both virulent and saprophytic stains of Mycobaterium. Human tubercle bacilli give a strong positive niacin test whereas other types give a negative test for niacin production.

	Optimum Temperature	Rate of Growth	Pigment	Guinea Pig Virulence
M. Tuberculosis	37º C	Eugonic	Positive	Positive
M. bovis	37º C	Dysgonic	Negative	Positive
M. avium	40-42º C	Rapid	Positive	Variable
Atypical AFB	25-37º C	Rapid	Positive	Negative

Table 4-6. Characteristics of the acid-fast bacilli (AFB).

a. **Mycobaterium Tuberculosis.** Strains of this organism usually exhibit growth within 12 to 25 days on Lowenstein-Jensen medium. Since luxuriant colonies are formed, they are termed eugonic ("growing luxuriantly"). The growth is dry, friable, nodular, rough, and buff-colored. After full development, typical colonies are 3 to 5 mm in diameter and possess flat irregular margins and rough, "cabbage head" centers. Whole colonies are easily detached from agar surface but are difficult to suspend in physiological saline. Occasional strains of M. tuberculosis are dysgonic (bad or poor growth) and must be different from M. bovis.

b. **Mycobacterium Bovis.** Colonies of bovine bacilli develop more slowly than human stains usually requiring from 25 to 40 days of incubation. The colonies are tiny, translucent, colorless, smooth, and pyramidal. Because there is slight growth as compared with colonies of human forms, they are termed dysgonic. The colonies adhere to the surface of the medium but are easily suspended in saline.

c. **Mycobacterium Avium.** The avian bacilli usually form colonies in 14 to 21 days. They are smooth viscid or butyrous (butter-like) and hemispherical. They may possess a faint pink or yellow pigment. Since the colonies are more rapidly formed and somewhat larger (1 to 2 mm in diameter) than those of the bovine type, they are referred to as eugonic. The growth is mucoid and tenacious, yet easily suspended in normal saline.

d. **Mycobacteria of Runyon Groups I-IV.** Mycobacteria other than M. tuberculosis and M. bovis are occasionally associated with both pulmonary and extrapulmonary diseases. These organisms consists of a number of acid-fast bacilli that have been variously described as "atypical," "anonymous," or "unclassified." Since 1959, these mycobacteria have been classified into four large groups called Runyon groups, which now include some well-defined species. Many of these forms produce a disease that is clinically similar to tuberculosis, but culturally they exhibit several features that set them apart from M. tuberculosis and M. bovis. These bacteria are acid-fast and are stained by the same procedure as the tubercle bacilli. The source of these organisms is human sputum and occasionally specimens from other sites. Morphologically, these bacteria resemble the tubercle bacilli, but they have other characteristics typical of their own species. The taxonomic position and the medical significance of many of these organisms are unsettled questions. It is clear that some produce disease closely resembling tuberculosis. These "anonymous" mycobacteria are divided into Runyon groups according to pigmentation and growth rate. Most workers refer to them as group number, groups I, II, III, and IV.

(1) Group I consists of photochromogens. The colonies of this group produce little or no pigment when grown in the dark. When exposed to light during their period of active growth their colonies develop a yellow pigmentation. This group requires complex media for satisfactory growth. The colonies grow faster than the tubercle bacilli, usually within 1 and 2 weeks. One member of this group, Mycobacterium kansasii, produces a human pulmonary disease indistinguishable clinically or histologically from tuberculosis.

(2) Group II consists of scotochromogens. Organisms in this group produce pigmented colonies that are yellow-orange in either light or dark. No growth is obtained on plain nutrient agar. Group II is a very heterogenous group that usually is not involved in human disease. Growth requires 1 or 2 weeks of incubation.

(3) Group III, the nonphotochromogens, is not affected by exposure of the growing colonies to light as occurs with groups I and II. It is said that group III is not pigmented. The true color is a buff or light tan and may be cream or ivory. These organisms grow much faster than the tubercle bacillus. Growth usually is observed in 1 or 2 weeks. Prominent members of this group are M. avium, discussed before, and M. intracellulare (the Battey bacillus). The Battey bacillus causes a lung disease in humans and resembles the avian species of the tubercle bacillus.

(4) Group IV, the rapid growers, contains organisms that are non-pigmented but grow rapidly on simple media. One member of this group, mycobacterium fortuitum, is associated with a progressive pulmonary disease. Growth on simple media may be observed in 2 to 4 days. One outstanding concern is the relative resistance of most "anonymous mycobacteria" to the primary antituberculosis drugs, such as streptomycin, isoniazid, and para-aminosalicylic acid.

4-46. SAPROPHYTIC MYCOBACTERIA

Saprophytic mycobacteria frequently occur in clinical specimens and must be differentiated with certainty from pathogenic forms. Acid- fast bacilli can be considered saprophytic only when they are not closely associated with lesions in man and fail to parasitize laboratory animals. It is desirable that all acid-fast isolates be inoculated to suitable laboratory animals before reaching a conclusion. Occasionally, even animal virulence studies will not conclusively exclude an organism from being a pathogen for the particular patient concerned. The saprophytic mycobacteria are composed of such species as Mycobaterium smegmatis, and M. phlei.

4-47. CONFIRMATORY PROCEDUES FOR MYCOBACTERIA

a. **Guinea Pig Virulence Test.** The presence of acid-fast bacilli in direct examination and typical colonies on culture is strong evidence of tuberculosis. Demonstration of specific lesions in laboratory animals is generally unnecessary for confirmation, but it is helpful in the detection of small numbers of tubercle bacilli, such as in cerebrospinal fluid. Very few bacilli are required to infect the guinea pig, and it is equally susceptible to infection with both human and bovine species.

(1) Two guinea pigs are usually inoculated simultaneously in the muscle tissue of the groin. A positive test shows an induration that appears at the site of inoculation and in 4 to 6 is usually accompanied by palpable lymph nodes. After 6 weeks, both animals may be infected animals will develop an intense local reaction that gradually becomes an open ulcer. Animals that are negative for this test will demonstrate only a slight erythema.

(2) Animals that show a positive test are sacrificed and the bacilli from the lesions are demonstrated by smears and cultures. The tubercle lesions are small millet-seed-like, buff-colored tubercles. They are commonly located in the spleen, lymph nodes, liver, kidneys, lungs, and site of inoculation.

b. **Referral.** Many clinical laboratories are ill equipped to perform animal pathogenicity tests. When facilities are lacking, cultures should be shipped to reference laboratories for confirmation. Specimens to be shipped for culture should be placed in equal volumes of trisodium phosphate solution.

4-48. CATALASE TEST (FOR ACID-FAST BACILLI)

a. **Principle.** Acid-fast bacilli produce the enzyme catalase. Catalase activity will be decreased when the bacilli become resistant to the therapeutic agent, isoniazid. The loss of catalase activity is also correlated with a weakening of virulence for the guinea pig. It is possible to subgroup acid-fast bacilli on the basis of their catalase activity at different temperatures and pH. Two methods are employed.

b. **Reagents.**

(1) 30 percent hydrogen.

(2) 10 percent Tween 80.

c. **Room Temperature Method.**

(1) Prepare a 1:1 mixture of 10 percent Tween 80 and 30 percent hydrogen peroxide.

(2) At room temperature, add 0.5 ml of this mixture to a slant containing Mycobacterium growth.

(3) Observe for bubbling.

d. **Temperature and pH Effects Method.**

(1) Add several loopfuls of Mycobacterium growth from a slant to 0.5 ml of phosphate buffer solution in a test tube.

(2) Incubate in a 68º C water bath for 20 to 30 minutes.

(3) Prepare a 1:1 mixture of 10 percent Tween 80 and 30 percent hydrogen peroxide.

(4) After incubation, add 0.5 ml of this mixture to the buffer-growth mixture.

(5) Observe for bubbling.

e. **Interpretation.**

(1) At room temperature most acid-fast bacilli are catalase positive. Those strains not exhibiting catalase activity are usually isoniazid-resistant, possessing little virulence for guinea pigs.

(2) At 68º C and pH 7.0, the catalase activity of human and bovine tubercle bacilli is selectively inactivated. Under the same conditions all other acid-fast bacilli are catalase positive.

4-49. NEUTRAL RED TEST (FOR ACID-FAST BACILLI)

a. **Principle.** Cells of virulent Mycobacterium suspended in barbital buffer solution are bound by the indicator methyl red, while avirulent strains are not affected.

b. **Reagents.**

(1). 50 percent methyl alcohol.

(2) 5 percent sodium chloride solution.

(3) 1 percent sodium barbital.

(4) 0.2 percent aqueous neutral red solution.

c. **Procedure.**

(1) Suspend several Mycobacterium colonies in 5 ml of 50 percent methyl alcohol.

(2) Incubate for one hour at 37º C. Centrifuge and discard the supernate. Repeat the washing procedure using another 5 ml of 50 percent methyl alcohol.

(3) After discarding the supernate, resuspend the sediment in 5 ml of freshly prepared barbital buffer (equal volumes of 5 percent sodium chloride and 1 percent sodium barbital).

(4) Add 1 ml of aqueous neutral red solution to the growth-buffer mixture. Allow to stand for 30 minutes at room temperature before reading.

d. **Interpretation.**

(1) Cells of virulent Mycobacterium will develop red color.

(2) Avirulent Mycobacterium will remain yellow in the alkaline barbital solution.

4-50. NIACIN TEST (FOR ACID-FAST BACILLI)

a. **Principle.** Mycobacterium tuberculosis produces niacin. Niacin will combine with cyanogen bromide and develop a yellow color.

b. **Reagents.**

(1) Sterile water.

(2) 95 percent ethyl alcohol.

(3) 10 percent cyanogen bromide, aqueous.

(4) 4 percent aniline, alcoholic.

c. **Procedure.**

(1) Add 0.5-1.0 ml of sterile water to a slant culture of acid-fast bacilli.

(2) Place culture tube at such an angle as to allow the water to layer over the colonies. This will extract any niacin present within 5 to 10 minutes.

(3) Transfer 0.5 ml of fluid from slant to a test tube. Add equal quantities of 4 percent aniline in ethyl alcohol and 10 percent aqueous cyanogen bromide.

CAUTION: Avoid inhalation of toxic cyanogen bromide fumes.

d. **Interpretation.** The presence of niacin is evidence by the immediate development of a yellow color throughout the mixture.

Continue with Exercises

EXERCISES, LESSON 4

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. For the diagnosis of gonorrhea in women, a swab obtained from the endocervical canal is ordinarily used to:

   a. Inoculate an MTM medium.

   b. Prepare a gram-stained smear.

   c. Perform sugar-fermentation reactions.

   d. Prepare a fluorescent antibody-stained smear.

2. In men, smears of urethral discharge for suspected gonorrhea should be stained with:

   a. Crystal violet.

   b. Gram stain.

   c. Ziehl-Neelsen stain.

   d. Giemsa stain.

3. Modified Thayer-Martin medium is selective for pathogenic:

   a. Neisseriae.

   b. Corynebacteria.

   c. Clostridia.

   d. Mycobacteria.

4. Bottled MTM medium is recommended only when specimens cannot be delivered to the laboratory or incubator:

   a. Within 30 hours.

   b. Within 36 hours.

   c. On the same day.

   d. Within 48 hours.

5. A CO_2 incubator for culturing gonococci should have a temperature of _____ a humidity of _____ and a CO_2 concentration of _____.

   a. 15º to 16º C, 10 percent to 30 percent, 25 to 30 percent.

   b. 25º to 26º C, 30 percent to 50 percent, 15 to 20 percent.

   c. 35º to 36º C, 50 percent to 70 percent, 5 to 10 percent.

   d. 45º to 46º C, 70 percent to 90 percent, 35 to 40 percent.

6. Which of the following tests is used as part of the presumptive identification of Neisseria species?

   a. Catalase.

   b. Coagulase.

   c. Oxidase.

   d. Optochin.

7. Which of the following must be performed on all oxidase positive colonies to confirm that they are Neisseria species?

   a. Catalase.

   b. Coagulase.

   c. Methylene blue stain.

   d. Gram stain.

8. Presumptive identification of Neisseria gonorrhea (other than obtained from anal or genital sites) in isolates should be confirmed by sugar fermentation or fluorescent antibody reactions when the specimen is:

    a. ANY OF THE BELOW.

    b. Blood.

    c. Synovial fluid.

9. Which of the following Neisseria species is generally glucose positive and maltose negative?

    a. Neisseria meningitides.

    b. Branhamella (Neisseria) catarrhalis.

    c. Neisseria gonorrhea.

    d. Neisseria sicca.

10. What isolate should have the following characteristics?

    Aerobic.
    Gram-negative cocci arranged in pairs and having a coffee bean-shaped. appearance.
    Grayish-white, small, hard colonies on blood agar.
    Blackening of colonies when tested with oxidase reagent.
    No production of acid from glucose, maltose, or sucrose.

    a. Bacillus anthracis.

    b. Klebsiella pneumoniae

    c. Branhamella (Neisseria) catarrhalis.

    d. Neisseria gonorrhea.

11. The usual portal of entry for the meningococcus is:

    a. The conjunctivas.

    b. The nasopharynx.

    c. Breaks in the skin.

    d. The urogenital system.

12. Which of the following enriched media is more useful for the isolation of the pathogenic Neisseria species?

    a. Nutrient agar.

    b. MTM.

    c. EMB.

13. Assume that you have isolated cocci that are gram negative, coffee bean shaped, and associated in pairs from the spinal fluid of a meningitis case. These cocci are probably:

    a. Streptococcus (Diplococcus) pneumoniae.

    b. Neisseria meningitides.

    c. Neisseria gonorrhea.

    d. Saprophytic neisseriae.

14. The saprophytic corynebacteria are known as:

    a. Diphtheria bacilli.

    b. Diphtheroids.

    c. Corynebacterium gravis.

    d. Corynebacterium mitis.

15. The characteristic metachromatic bodies corynebacterium diphtheriae are called:

    a. Koch-Weeks bodies.

    b. Donovan bodies.

    c. Babes-Ernst granules.

    d. Widal bodies.

16. A smear from a colony of Corynebacterium diphtheriae should show bacilli that are:

    a. Gram of positive, spore forming.

    b. Gram negative, spore forming

    c. Gram positive, in Chinese-letter patterns.

    d. Gram negative, in Chinese-letter patterns.

17. Loeffler's serum slants and potassium tellurite agar palates are used especially for the isolation of organisms in suspected cases of:

    a. Tuberculosis.

    b. Diphtheria.

    c. Whooping cough.

    d. Anthrax.

18. The entry of Corynebacterium diphtheriae is usually through:

    a. The unbroken skin.

    b. An open wound.

    c. The nasopharynx.

    d. The conjunctivas.

19. The presence of a clotting film, or "pseudomembrane" in the throat is symptomatic of infection with:

    a. Bacillus anthracis.

    b. Mycobacterium tuberculosis.

    c. Clostridium botulinum.

    d. Corynebacterium diphtheriae.

20. The schick test is a test for the presence of:

    a. Diphtheria antitoxin.

    b. Diphtheria toxoid.

    c. Tuberculosis toxin-antitoxin in the tissues.

    d. Tuberculosis antitoxin.

21. Strains of corynebacteria that cause diphtheria can be distinguished from most other strains by demonstrating:

    a. Production *of* diphtheria toxin.

    b. Production of catalase, reduction of nitrate to nitrite, and inability to decompose gelatin or urea.

    c. Fermentation of glucose and sucrose with no gas and inability to ferment maltose.

    d. The morphology of nonmotile, nonsporogenous, nonencapsulated, pleomorphic, gram-positive bacilli.

22. In the in vivo test used to determine the pathogenicity of a suspected strain of Corynebacterium diphtheriae, the control guinea pig is given a protective dose of:

    a. Epinephrine.

    b. Penicillin.

    c. Diphtheria antitoxin.

    d. Diphtheria toxoid.

23. What isolate should have the following characteristics?

    Aerobic and nonmotile.
    Gram-positive bacilli, variable in size, some club-shaped, tending to a Chinese-letter arrangement.
    On blood agar--small white, dull colonies showing no hemolysis.
    On tellurite medium-gray colonies.
    Non-toxigenic.
    Production of acid from glucose and sucrose.

    a. Shigella species.

    b. Pseudomonas aeruginosa.

    c. Corynebacterium xerosis.

    d. Corynebacterium diphtheriae.

24. Listeria monocytogenes is a:

    a. Gram-positive bacillus.

    b. Gram-positive coccus.

    c. Gram-negative bacillus.

    d. Gram-negative coccus.

25. An alpha-hemolytic gram-positive rod gives a negative catalase test. This aerobic organism fails to produce spores and could be:

    a. Listeria.

    b. Streptococcus.

    c. Corynebacterium.

    d. Erysipelothrix.

26. The genus Bacillus includes only one important pathogenic species. It is:

    a. Bacillus subtilis.

    b. Bacillus cereus.

    c. Bacillus megaterium,

    d. Bacillus anthracis.

27. Bacillus subtilis differs from Bacillus anthracis in that Bacillus subtilis is a:

    a. Motile and nonencapsulated organism.

    b. Nonmotile and encapsulated organism.

    c. Gram-positive bacillus with square ends.

    d. Gram-negative bacillus with rounded ends.

28. Which of the following organisms produces spores?

    a. Mycobaterium tuberculosis,

    b. Mycobacterium leprae

    c. Bacillus anthracis.

    d. Corynebacterium diphtheriae.

29. An important basis for differentiation of Bacillus anthracis from the pseudoanthrax bacilli is that B. anthracis colonies are:

    a. Very hemolytic on blood agar.

    b. Nonencapsulated.

    c. Nonhemolytic or very weakly hemolytic on blood agar.

    d. Motile.

30. Organisms causing human anthrax usually enter through the

    a. ANY OF THE BELOW.

    b. Skin.

    c. Lungs.

    d. Gastrointestinal system.

31. Which of the following genera consists of strict anaerobes?

    a. Mycobacterium.

    b. Corynebacterium.

    c. Clostridium.

    d. Bacillus.

32. Clostridium tetani gives the appearance of a microscopic drumstick due to:

    a. Bipolar staining.

    b. Metachromatic granules.

    c. Round terminal spores.

    d. Oval central spores.

33. Clostridium perfringens is associated with all of the following EXCEPT:

    a. Anaerobiosis.

    b. Nonmotility.

    c. Consistent spore formation.

    d. Dirty wounds.

34. Which of the following organisms is nonmotile?

    a. Clostridium tetani.

    b. Clostridium septicum.

    c. Clostridium botulinum.

    d. Clostridium perfringens.

35. All of the following describe Clostridia EXCEPT:

    a. Anaerobic organisms.

    b. Gram-positive rods.

    c. Soil inhabitants.

    d. Producers of powerful endotoxin.

    e. Spore formers.

36. Clostridium tetani is pathogenic because the organism:

    a. Is anaerobic.

    b. Forms spores.

    c. Produces a potent neurotoxin (nerve poison).

    d. Is highly invasive.

    e. Produces irritant to symbiotic scavengers.

37. The flora of gas gangrene may include:

    a. ANY OF THE BELOW.

    b. Cl. Novyi.

    c. Cl. Septicum.

    d. Cl. perfringens.

38. The organism most commonly associated with gas gangrene is:

    a. Clostridium botulinum.

    b. Clostridium perfringens.

    c. Salmonella typhi.

    d. A Shigella species.

39. Clostridium botulinum would most likely be isolated from which of the following specimens?

    a. Blood.

    b. Contaminated food.

    c. Feces.

    d. Urine.

40. The mycobacteria are designated acid-fast because:

    a. They are easily stained with acid dyes.

    b. They cannot be stained with acid dyes.

    c. Once stained they are easily decolorized by acid-alcohol.

    d. Once stained they are not easily decolorized by acid-alcohol.

41. Which of the following organisms is NOT grown routinely in vitro (outside the living host)?

    a. Mycobacterium bovis.

    b. Mycobacterium leprae.

    c. Bacillus anthracis.

    d. Diptheroids.

42. Which of the following species is almost always responsible for tubercular infection of the lungs of humans?

    a. Mycobacterium tuberculosis.

    b. Mycobacterium bovis.

    c. Mycobacterium avium.

    d. Mycobacterium leprae.

43. What reagent may be used to prepare a concentrated sputum specimen to be examined microscopically for Mycobacterium?

    a. 5 percent sodium hypochlorite

    b. 4 percent sodium chloride.

    c. 95 percent ethyl alcohol.

    d. Pepsin.

44. Mycobacterium leprae resembles M. tuberculosis in its:

    a. ALL OF THE BELOW.

    b. Gram reaction.

    c. Size and shape.

    d. Acid-fast staining properties.

45. Digestion of a sputum specimen for culture of Mycobacterium tuberculosis is accomplished with a reagent containing:

    a. N-acetyl-L-cysteine.

    b. Sodium hypochlorite.

    c. Boric acid.

    d. Pepsin.

46. To cultivate tubercle bacilli, it is wise to use either Lowenstein-Jensen medium (or Petragnani's medium) and:

    a. Bordet-Gengou medium.

    b. 7H11 agar.

    c. Thayer-Martin agar.

    d. Triple sugar iron agar.

47. Colonies of Mycobacterium tuberculosis on Lowenstein-Jensen medium are:

    a. Tiny, pale, and pyramidal.

    b. Hemispherical and smooth, with a faint yellow pigmentation.

    c. Dry, friable, somewhat rough, buff-colored, with cabbage head centers and flat irregular margins.

    d. Small, glistening, translucent or transparent, and soft in texture.

48. Mycobacteria other than M. tuberculosis and M. bovis have been classified into four large groups called Runyon groups according to:

    a. Human pathogenicity.

    b. Niacin production.

    c. Acid-fast staining characteristics.

    d. Pigmentation and growth rate.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES, LESSON 4

1. a. (para 4-4a(1))
2. b (para 4-5a(1))
3. a (para 4-9)
4. c (para 4-9c)
5. c (para 4-13a(1)(2))
6. c (paras 4-14b; 4-21a)
7. d (para 4-14c)
8. a (paras 4-6a; 4-14; table 4-1)
9. c (table 4-1)
10. c (paras 4-2; 4-14; table 4-1)
11. b (para 4-18)
12. b (para 4-19)
13. b (paras 4-4 - 4-6; 4-18 - 4-20)
14. b (para 4-22a)
15. c (para 4-23a)
16. c (para 4-23a)
17. b (paras 4-24a; 4-26a(2))
18. c (para 4-25b)
19. d (para 4-25b)
20. a (para 4-25b)
21. a (para 4-26a(3); table 4-3
22. c (para 4-26b; table 4-3)

23. c (para 4-23; table 4-3)

24. c (para 4-27)

25. d (para 4-28; Figure. 3-1)

26. d (para 4-29)

27. a (para 4-29)

28. c (para 4-29; 4-30)

29. c (para 4-31)

30. a (para 4-32a(2), b(1))

31. c (para 4-34a; 4-35)

32. c (para 4-34a; table 4-5)

33. c (paras 4-34a; c; 4-37c(1)(2); Table 4-5)

34. d (para 4-34c; table 4-5)

35. d (paras 4-34; 4-37)

36. c (para 4-37a)

37. a (para 4-37c)(1))

38. b (para 4-37c(1))

39. b (para 4-38a(2))

40. d (para 4-22d)

41. b (para 4-39)

42. a (para 4-40a)

43. a (para 4-41e)

44. a (para 4-42c)

45. a (para 4-43a)

46. b (paras 4-43c(11); 4-44b)

47. c (para 4-45a)

48. d (para 4-45d)

End of Lesson 4

LESSON ASSIGNMENT

LESSON 5	Enterobacteriaceae.
LESSON ASSIGNMENT	Paragraph 5-1 through 5-35.
LESSON OBJECTIVES	Upon completion of this lesson, you should be able to:

5-1. Identify descriptive features of the family Enterobacteriaceae.

5-2. Associate names of genera and species of Enterobacteriaceae with types of diseases they may cause.

5-3. Associate specific types of enteric media with their uses, methods of handling, and interpretation of typical reactions.

5-4. Associate names of Enterobacteriaceae with their typical gram morphology, colony morphology, and biochemical reactions.

5-5. Given two different categories of Enterobacteriaceae, identify tests useful in differentiating them.

SUGGESTION: After reading and studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

ENTEROBACTERIACEAE

Section I. INTRODUCTION

5-1. DEFINITION

The family Enterobacteriaceae consists of gram-negative, aerobic (facultatively anaerobic), nonsporogenous bacilli that grow well on artificial media. They may be motile or nonmotile, but motile forms must be peritrichous, that is, possess flagella distributed over the entire surface of the bacterial cell. Members of the family reduce nitrates to nitrites, ferment glucose with the production of acid or of acid and gas, do not produce indophenol-oxidase, and do not liquefy alginate. Pectobacterium is the only genus of the family that liquefies pectate. The genera in the family Enterobacteriaceae are Escherichia, Shigella, Edwardsiella, Salmonella Arizona, Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Providencia, Erwinia, Pectobacterium, and Yersinia.

5-2. NORMAL FLORA AND GENERAL IDENTIFICATION OF SPECIES

Many of these bacteria are normally present in the intestinal tract as part of the normal flora. It becomes the task of the bacteriology laboratory to differentiate between the gram-negative, glucose-fermenting bacilli that are normally present in the intestines, and those that are considered to be pathogens. It must be remembered that the reactions and classifications given in this study guide are broadly accepted, but due to the very nature of the subject itself, different textbooks and authors may vary on certain points and the specific reactions of a particular organism. To completely identify any one of these enteric bacteria, all characteristics of the bacterium must be established to include colony morphology on differential and selective media, numerous biochemical patterns, and often serological characteristics.

5-3. MORPHOLOGY AND CULTURE

The enteric gram-negative rods range from 1 to 4 microns in length and from 0.4 to 0.8 microns in breadth. A few longer, filamentous cells may be exhibited by any of the species. The organisms possess no typical cellular arrangement and may be observed singly, in pairs, in clumps, and occasionally in short chains. Microscopic morphology is, therefore, of little diagnostic value. The majority of the enteric gram-negative rods are actively motile. The enteric bacilli grow well on ordinary nutrient media. Most species are facultative anaerobes. Although the majority of these organisms usually yield good growth between 20º C and 40º C, 37º C is optimum for most species, especially the pathogens. A medium of approximately neutral pH is most favorable for growth of all enteric bacilli. A great variety of culture media may be employed for isolation and identification of pathogenic enteric bacilli in fecal specimens. This includes the use of differential, selective and inhibitory plating media as well as selective and enrichment broths.

Section II. PATHOGENICITY OF ENTEROBACTERIACEAE

5-4. PATHOGENICITY OF THE GENUS ESCHERICHIA

Escherichia coli is one of the most abundant species of bacteria represented in the normal intestinal tract. In this region, the organism contributes to normal function and nutrition. E. coli and other enteric saprophytes become pathogenic when introduced into tissues outside the intestinal tract, especially the urinary and biliary tracts, peritoneum, or meninges. E. coli more frequently invades the urinary tract and is the most common cause of cystitis. The organism has also been isolated from local infections such as conjunctivitis. E. coli may also be the cause of septicemia. A number of E. coli serotypes have been associated with infant diarrhea, and when E. coli is isolated from pediatric patients, it should always be serotyped.

5-5. PATHOGENICITY OF THE GENUS KLEBSIELLA

Klebsiella pneumoniae (Friedlander's bacillus) is isolated with some frequency from the upper respiratory and intestinal tracts of normal individuals and is responsible for approximately two percent of the bacterial pneumonias. Pulmonary infections are characterized by extensive hemorrhagic consolidation of the lobes. The fatality rate is high in untreated cases. Klebsiella species are frequently isolated from various upper respiratory tract infections, although their presence, in many instances, is probably that of secondary invaders. The organisms have definitely been responsible for suppurative abscesses of the other visceral tissue.

5-6. PATHOGENICITY OF THE GENUS ENTEROBACTER

Several species of Enterobacter--E. cloacae, E. liquefaciens, E. aerogenes, and E. hafniae--have been recognized and exhibit a pathogenicity similar to Escherichia. Species of Enterobacter are isolated frequently in cases of septicemia and urinary tract infections.

5-7. PATHOGENICITY OF THE GENUS PROTEUS

Of the genus Proteus, four species are recognized--Proteus vulagaris, P. mirabilis, M. morganii, and P. rettgeri. Although these organisms are primarily free-living in water, soil, and sewage, they are frequently isolated from fecal specimens of normal individuals. Morganella morganii has been responsible for diarrhea of infants and children. Proteus species often cause human infections and usually do so when introduced into tissues other than the normal intestinal tract. In this connection, Proteus species rank next to E. coli as the etiological agent of cystitis. These organisms are also encountered frequently in eye and ear infections and occasionally in pleurisy, peritonitis, and suppurative abscesses in many areas of the body. Proteus is commonly associated with other bacteria in purulent wounds and may contribute to the severity of such infections.

5-8. PATHOGENICITY OF THE GENUS SALMONELLA

It is important to remember that all salmonellae are potential pathogens and may produce enteric fever, septicemia, or gastroenteritis. Such infections often originate from ingestion of contaminated food or drink.

a. **Enteric Fevers.** The enteric fevers consist of typhoid fever and paratyphoid fever. Salmonella typhi is responsible for typhoid fever while S. paratyphi A, S. paratyphi B, and others are most often encountered in paratyphoid fever. Of these salmonellae, S. paratyphi A and S. paratyphi C are only occasionally isolated in the United States. In enteric fevers, the ingested organisms enter the small intestine, spread through the intestinal lymphatics to the thoracic duct and enter the blood stream. The resultant septicemia distributes the infection to many organs including the kidney, intestines, liver, gallbladder, and other tissues. Infections are characterized by an insidious onset, with low-grade fever that ultimately becomes quite elevated during the bacteremic phase. Blood cultures are usually positive only during the first and second week of infection. Stool and urine cultures usually fail to yield the responsible Salmonella species until the third week. The duration of typhoid fever and paratyphoid fever is usually several weeks. Salmonella infections that result in septicemia are often due to Salmonella choleraesuis. The onset of symptoms is abrupt since blood stream invasion occurs within a short period of time following oral ingestion of the organism. This is accompanied by a rapid rise in temperature that spikes during the height of infection. Wide distribution of the organisms results in focal suppuration and abscess formation in various tissues. Meningitis, osteomyelitis, endocarditis, and pneumonia are known complications of such infections. Blood cultures are most often positive when taken during the height of the fever.

b. **Gastroenteritis.** Of the many Salmonella, species that produce acute gastroenteritis in man, S. typhimurium is the most frequent causative agent. Salmonella enteritidis is possibly the second most common cause. S. choleraesuis has also been implicated in gastroenteritis but to a lesser extent than either of the two previously mentioned species. Infections are characterized by fairly sudden onset (15 to 24 hours' incubation), and rather severe gastrointestinal distress with vomiting, diarrhea, and slight elevation of temperature. Recovery is rapid (1 to 3 days) since the intestinal tract is not usually invaded by the organisms. Symptoms result from the irritative action of acids and endotoxin upon the intestinal mucosa. The acids are formed by fermentation of carbohydrates by the responsible organisms. Endotoxins are released following death and cellular lysis of the etiologic agent. Only very rarely do infections develop into septicemia. Outbreaks of gastroenteritis are usually linked with the consumption of certain foods and are often referred to as "food poisoning." Diseases usually originate from unsuspected subclinical cases, convalescent carriers, or healthy permanent carriers who harbor the organisms in their intestine, gallbladder, or the urinary tract. Such individuals may contaminate food or drink either directly or indirectly. The salmonellae produce no exotoxins. Upon death and lysis of the cells, endotoxins are released which largely account for the disease symptoms of man.

5-9. PATHOGENICITY OF THE GENUS SHIGELLA

Shigellae are the cause of bacillary dysentery. Infections are usually limited to the gastrointestinal tract. The disease process is essentially an inflammation of the mucous membrane of the large intestine and terminal ileum that leads to necrosis and superficial ulceration. Symptoms occur within 1 to 2 days following ingestion of contaminated food or drink. The illness is characterized by sudden onset of abdominal pains, cramps, diarrhea, and fever. The intense irritation of the bowel is due to the release of somatic endotoxin upon autolysis of the Shigella species. Infections from S. dysenteriae are more severe because, in addition to the endotoxin substance, an exotoxin (neurotoxin) is produced which causes paralytic symptoms. Infections from exotoxin-producing strains of S. dysenteriae are relatively frequent in India, Japan, China, and other parts of Asia. Although some individuals recover quickly from bacillary dysentery and pass infectious bacilli in stools for only a short period, others become chronic carriers (ulcerative colitis) and may suffer frequent relapses of the disease. The latter serve as a reservoir of infection.

Section III. ENTERIC MEDIA

5-10. CATEGORIES OF ENTERIC MEDIA

Before you can expect to isolate and tentatively identify members of the enteric bacteria, you must have some knowledge of the different media that are used for this purpose. Generally speaking, enteric media can be divided into three categories.

a. Differential media are designed to point out differences in bacteria on the basis of their metabolism.

b. Selective media are designed to select certain potential pathogens from among nonpathogenic bacteria.

c. Enrichment media are designed to help enrich or promote the growth and recovery of certain types of pathogenic bacteria from clinical specimens, such as feces, which contain large numbers of saprophytic enteric bacteria.

5-11. LACTOSE FERMENTATION

The initial division of bacteria comprising the enterics is separated most conveniently using, as a reference point, the ability to ferment the sugar lactose. In this division, three areas of interest emerge:

a. The lactose fermenters include those enteric bacteria that are able to ferment lactose with the production of gas within 24 hours. The enteric bacteria exhibiting this characteristic are known as the "coliforms," and they are usually found as saprophytes in the intestinal tract. The coliforms do not usually present a medical problem except in pediatric cases.

b. The late lactose fermenters are able to ferment lactose after prolonged periods of incubation, usually after 48 hours. These enteric bacteria are generally referred to as the "paracolons." They are of interest because they exhibit characteristics of both the coliforms and the pathogens, and must be distinguished from them. The exception is Shigella sonnei that may show delayed (4 to 7 days) lactose fermentation.

c. Finally, the lactose nonfermenters represent the third area of interest as regards the ability to ferment lactose. These gram-negative enteric bacilli are usually unable to ferment lactose and include most of the pathogens and some saprophytic bacteria that are usually present in the intestinal tract as normal flora and must be distinguished from pathogens.

5-12. DIFFERENTIAL MEDIA

The differential media are designed to distinguish between colonies of lactose fermenters and lactose nonfermenters. Some of the differential (or isolation) media commonly used are eosin-methylene blue (EMB) agar, MacConkey's agar, and deoxycholate agars. These media contain certain carbohydrates, indicators, and chemicals that are inhibitory to a large number of the gram-positive bacteria that so often overgrow the relatively few pathogenic bacteria.

a. **EMB Agar.** Eosin-methylene blue (EMB) agar contains the dyes eosin and methylene as well as the carbohydrates lactose-and sucrose. The dyes act as inhibitors of most gram-positive bacteria and also as indicators of those bacteria capable of fermenting lactose. Colonies of lactose fermenters appear as dark-colored colonies while those of lactose nonfermenters appear as translucent or colorless colonies. EMB agar is particularly valuable in identifying Escherichia coli. On EMB, E.coli produces a very discrete and distinctive colony that has a green metallic sheen. EMB is always sterilized by autoclaving.

b. **MacConkey's Agar.** MacConkey's agar is a differential medium that also distinguishes between lactose fermenters and lactose nonfermenters. Colonies of lactose fermenters appear red or pink, while colonies of lactose nonfermenters are translucent or colorless. MacConkey's agar contains the pH indicator, neutral red, which gives a red color under acid conditions. The growth of gram-positive bacteria is inhibited on MacConkey's agar because of the presence of bile salts and the dye crystal violet. MacConkey's agar is also sterilized by autoclaving.

c. **Deoxycholate Agar.** Deoxycholate agar is very similar to MacConkey's agar and also contains the pH indicator neutral red and distinguishes between lactose fermenters and lactose nonfermenters. The colony appearance is very similar to that produced on MacConkey's agar. Deoxycholate agar is prepared by heating to the boiling point to dissolve the agar; however, it is not autoclaved to sterilize and for this reason it is very suitable for use as a pour plate.

5-13. SELECTIVE MEDIA

a. The selective media most commonly used are Salmonella-Shigella agar, deoxycholate citrate agar, brilliant green agar, and bismuth sulfite agar. These media select certain potential pathogenic enteric bacteria from clinical specimens because of the presence of inhibitory substances that inhibit the growth of gram-positive bacteria and retard the growth of coliforms. These inhibitory substances include bile salts and brilliant green dye.

b. Salmonella-Shigella (SS) agar also contains the pH indicator neutral red and the carbohydrate lactose. When colonies of "coliforms" (lactose fermenters) appear, they are red or pink in color. Colonies of lactose nonfermenters usually appear colorless or translucent. Although Salmonella-Shigella agar is usually classified as a selective medium, it differentiates between colonies of lactose fermenters and lactose nonfermenters. Incorporated in this selective medium are the salts, ferric citrate and sodium thiosulfate. The presence of these salts provides the medium with an indicator of hydrogen sulfide production. The colonies of bacteria producing hydrogen sulfide may have blackened centers, which is the result of the precipitation of ferric sulfide. The precipitate ferric sulfide results when hydrogen sulfide comes in contact with the ferric ion. SS agar is not prepared by sterilization, but it is heated until in solution, then allowed to cool, and poured into plates.

c. Sterilization is not required for SS agar since this highly selective medium contains such strong inhibitors. Brilliant green agar is sterilized by autoclaving. However, deoxycholate citrate agar and bismuth sulfite agar must only be heated sufficiently to dissolve the agar and not autoclaved, as excessive heating will destroy their selectivity.

d. All of the selective media mentioned serve the same general purpose, to inhibit many of the coliform bacilli, and especially Escherichia and Proteus strains, while permitting the growth of most Salmonella and some Shigella organisms. Most Salmonella and Shigella species, along with slow or non-lactose-fermenting organisms, generally form colorless colonies. However, some species may appear as black or greenish colonies on certain media as already mentioned, and the lactose-fermenting organisms, which are not inhibited, grow as pink or red colonies.

5-14. ENRICHED MEDIA

Enriched media are generally employed for the recovery of enteric pathogens from specimens that might contain few enteric pathogens but many enteric saprophytes or coliform organisms. By the use of these media, enteric saprophytes are inhibited while the growth of enteric is not inhibited. Consequently the recovery of enteric pathogens is enhanced.

a. **Selenite-F Broth.** Selenite-F broth is an enriched medium that contains the inhibitor, selenite. In this medium gram-positive bacteria and coliform bacteria are inhibited. When selenite-F broth is used as an enrichment medium, it is essential that subculturing be done no later than 18 hours after inoculation of the medium since coliform bacteria will be inhibited only for a period of about 18 hours following inoculation. Selenite-F broth is prepared by boiling or by use of free- flowing steam. It must not be sterilized by autoclaving.

b. **Tetrathionate Broth.** Tetrathionate broth is an enriched medium that is especially valuable for recovery of Salmonella. This medium contains bile salts, iodine, and brilliant green as inhibitors. Gram-positive bacteria, coliforms, and Shigella species are generally inhibited in this highly inhibitory medium.

5-15. TUBED DIFFERENTIAL MEDIA

All of the media mentioned in this chapter so far except the enrichment media are utilized as streak plates or pour plates. Kligler's Iron Agar (KIA) and Triple Sugar Iron Agar (TSI) are tubed differential media having a slant and a butt. The slant represents an aerobic environment; the butt represents an anaerobic environment.

a. **Medium Contents.**

(1) KIA contains two sugars and two indicator systems. Glucose is present in 0.1 percent concentration and lactose is present in 1.0 percent concentration. Phenol red is present as the pH indicator, and it gives a yellow color under acid conditions and a red color under alkaline conditions. Hydrogen sulfide production is indicated by the formation of the black precipitate, ferric sulfide. Hydrogen sulfide is formed as a result of action upon the salts, ferric ammonium citrate and sodium thiosulfate.

(2) Triple Sugar Iron Agar (TSI) is exactly the same formula as KIA, but with the addition of 1.0 percent sucrose. The glucose concentration is one-tenth the concentration of the lactose and sucrose to enable the detection of glucose fermentation alone. The small amount of acid produced by the fermentation of the glucose is oxidized so rapidly in the slant that the slant either remains or reverts back to alkaline (red), whereas the lower-oxygen tension in the butt retains and maintains an acid (yellow) reaction. Because of this situation that necessitates the free exchange of air with the slant, a tightly stoppered or screw-capped tube creates an acid condition that involves the slant and in this way may give misleading reactions of the medium.

(3) Because of the added advantage of three sugars, TSI is usually preferred to KIA. Table 5-1 gives the reactions that are possible using TSI and the interpretation of each reaction, as well as the organisms that would be the most likely suspects.

	Reaction	Carbohydrate Fermentation	Suspected Organisms
Slant Butt H_2S Gas	Alkaline (R) Alkaline (R) – –	No carbohydrate fermentation	*Pseudomonas* *Alcaligenes* *Herellea* (see chapter 10)
Slant Butt H_2S Gas	Acid (Y) Acid (Y) – +	Lactose and/or sucrose fermented Glucose fermented	*Escherichia* *Enterobacter-Klebsiella* *Proteus* *Providencia* Intermediate coliforms
Slant Butt H_2S Gas	Acid (Y) Acid (Y) + +	Lactose and/or sucrose fermented Glucose fermented	*Citrobacter* *Arizona*
Slant Butt H_2S Gas	Alkaline (R) Acid (Y) – –	Lactose and sucrose not fermented Glucose fermented	*Providencia* *Proteus* *Serratia* *Shigella* *Salmonella*
Slant Butt H_2S Gas	Alkaline (R) Acid (Y) + +	Lactose and sucrose not fermented Glucose fermented	*Proteus* *Citrobacter* (certain types) *Salmonella* *Arizona* (certain types) *Edwardsiella*

Table 5-1. Interpretations of TSI agar reactions.

b. **Medium Interpretation.**

(1) Following inoculation and incubation of the organism on TS1 medium, sugar fermentation is evidenced by conversion of all or portions of the medium from orange-red to yellow. The acid end products of such fermentations convert phenol red to its yellow acid state. Those organisms failing to ferment any of the sugars produce no change in the medium or convert it to a deeper red color. The latter reaction is a result of the action of alkaline metabolic end products upon phenol red.

(2) TSI agar also contains sodium thiosulfate and ferrous ammonium sulfate to serve as indicators of hydrogen sulfide production. Organisms capable of producing hydrogen sulfide do so through the utilization of sodium thiosulfate in an acid environment. Hydrogen sulfide formed in this manner reacts with ferrous ammonium sulfate to yield ferrous sulfide. The latter substance accumulates as a black precipitate either immediately under the base of the slant or deeper in the butt of the TSI agar.

(3) After an 18 to 24 hour incubation period, the TSI medium is examined for the following reactions:

(a) Lactose or sucrose fermentation results in an acid (yellow) reaction throughout the medium.

(b) Fermentation of only glucose results in an acid (yellow) reaction in the butt of the agar medium with an alkaline (dark red) slant.

(c) If no sugars are fermented, an alkaline (dark red) with an orange-red butt is usually observed.

(d) Gas production in TSI agar from lactose, sucrose, or glucose fermentation results in bubble formation or splitting of the medium in the butt of the tube.

(e) Hydrogen sulfide production results in a blackening in the butt or the area of the medium just under the base of the slant.

c. **Identification.** After interpretation of TSI agar reactions, it is necessary to use numerous biochemical fermentation tests and serotyping to be certain of just which organisms you have.

5-16. ISOLATION OF ENTERIC PATHOGENS FROM FECAL SPECIMENS

In attempting to isolate enteric pathogens from fecal specimens, one or two procedures may be used. One method is to plate the specimen directly to any of the differential and selective media. The use of bismuth sulfite agar is highly recommended when Salmonella typhi is suspected, since bismuth sulfite inhibits the growth of most other organisms and promotes the growth of Salmonella typhi, producing a highly characteristic colony. Eosin-methylene-blue agar, MacConkey's agar, and SS agar are probably the most commonly used for the direct plating of fecal specimens. In the indirect procedure for the isolation of the enteric pathogens, either selenite-F or tetrathionate broth are inoculated with a fecal sample for enrichment. If selenite-F broth is used, the culture should be incubated for 8 to 12 hours at 37º C, and then streaked to EMS, MacConkey's, SS, bismuth sulfite, etc. If tetrathionate broth is used, the culture should be incubated for 12 to 24 hours at 37º C, and then streaked to EMS, MacConkey's etc.

Section IV. PRELIMINARY SCREENING OF CULTURES FOR ENTEROBACTERIACEAE

5-17. COLONY CHARACTERISTICS

Medical laboratory specialists working in the bacteriology section I become familiar with the colony characteristics of enteric bacilli on blood agar as well as on commonly used plating media for stool examination. Enteric organisms cultivated on blood agar usually reveal large, smooth, shiny, circular, raised colonies which mayor may not be hemolytic or pigmented. Proteus species often exhibit swarming. The colony characteristics of enteric species cultivated on commonly used enteric media are described in table 5-2 as they appear when cultivated for 16 to 24 hours at 37º C. Colorless colonies on differential and selective media indicate the organism is a lactose nonfermenter and, thus, a possible pathogen which must be identified by further studies. (Delayed lactose fermenters will also appear colorless at this stage.) Colored colonies indicate the organism is a lactose fermenter. Organisms producing colored colonies on enteric media are usually nonpathogenic for adults, providing these organisms have been isolated from their normal habitat, the intestinal tract. Certain lactose-fermenting enteric organisms may, however, be etiological agents in cases of infant diarrhea.

Organism	Differential media		Selective media	Inhibitor media	
	EMB agar	MacConkey's or deoxycholate agars	SS or deoxycholate citrate agars	Bismuth sulfite agar	Brilliant green agar
Escherichia coli	Large, regular colonies; maroon, purple or black centers; green metallic sheen.	Large, regular brickred colonies surrounded by a red zone.	Inhibited or slight growth of pink to red colonies.	Usually inhibited; occasional growth of small, black, green or brown colonies.	Inhibited or growth of yellow-green colonies with intense yellow zone.
Citrobacter	Translucent; greenish metallic sheen.	Uncolored, transparent; red	Black centered; clear periphery	Black; green-brown	Same as *E. coli.*
Enterobacter	Large, shiny, mucoid colonies; pink center and colorless periphery; no sheen.	Large, shiny, mucoid colonies; red center and colorless periphery.	Inhibited, or slight growth of pink to red colonies.	Same as *E. coli.*	Inhibited or growth of mucoid yellow-green colonies with yellow zone.
Klebsiella	Large, slimy, irregular colonies; pink center and colorless periphery; very mucoid.	Large, slimy, viscous confluent growth; red centered colonies.	Inhibited, or slight growth of mucoid, pink to red colonies.	Same as *E. coli.*	Same as *Enterobacter.*
Proteus	Small, colorless, discrete colonies or "swarming" spreading growth (foul odor).	Small, colorless, discrete colonies; occasional spreading growth.	Small, discrete colorless colonies.	Flat or slightly raised green colonies.	Inhibited or red colonies with intense red zones.
Pseudomonas	Small, translucent, colorless colonies, often irregular (sweet aromatic odor).	Similar to growth on EMB agar.	Small, regular, translucent, colorless colonies.	Inhibited	Inhibited or pink-white, translucent colonies with red zone.

Table 5-2. Colony characteristics of enteric bacilli on enteric media (continued).

Organism	Differential media		Selective media	Inhibitor media	
	EMB agar	MacConkey's or deoxycholate agars	SS or deoxycholate citrate agar	Bismuth sulfite agar	Brilliant green agar
Alcaligenes faecalis	Small, regular, translucent, colorless colonies.	Similar to growth on EMB agar.	Inhibited or colorless colonies.	Inhibited	Same as *Pseudomonas*
Arizona	Translucent; greenish metallic sheen	Colorless transparent; red	Black centered; clear periphery	Black; green brown	Inhibited or pink-white opaque colonies with brilliant red zone.
Salmonella	Small, regular, translucent, colorless colonies.	Small, regular, translucent, colorless colonies.	Small, regular colorless colonies opaque, translucent or transparent.	Flat or slightly raised colonies; some black, others green or dark brown.	Slightly pink-white, opaque colonies with brilliant red zone.
Shigella	Regular, translucent, colorless colonies; smaller than *Salmonella* spp.	Same as *Salmonella* spp. but smaller.	Same as *Salmonella* spp., but smaller; some strains inhibited.	Usually inhibited; some spp. form brownish, raised colonies with depressed centers.	Inhibited.

Note: Certain *Proteus* and *Salmonella* species may produce colorless colonies with black centers on SS agar. A bluish water soluble pigment may or may not be evidenced with Pseudomonas aeruginosa. Brilliant green agar is not suitable for growth of Salmonella typhi or Shigella spp.

Table 5-2. Colony characteristics of enteric bacilli on enteric media (concluded)

5-18. USE OF LESS INHIBITORY MEDIA

In attempting to isolate enteropathogenic Eschrichia coli, Klebsiella, or Citrobacter from fecal material, the use of tetrathionate and selenite-F broths for enrichment is dispensed with, since both are inhibitory for most strains of these groups. Because of such situations, the less inhibitory media (EMB and MacConkey's agar) are usually used for primary isolation by the direct plating procedure in lieu of the more selective media which are intended to inhibit the majority of these strains. All colonies which exhibit a lactose-nonfermenting appearance on the isolation plates, either from direct streaking or after streaking from an enrichment, should be suspected as possible enteric pathogens and further studies are indicated to establish or rule out the presence of enteric pathogens as opposed to normal flora which are also lactose negative or are late lactose fermenters. Suspected colonies from the isolation plates should be carefully transferred to TSI slants.

a. **TSI Slants.**

(1) Special care must be taken to insure that only pure cultures are transferred to a TSI slant, because if more than one type of organism is introduced into the TSI slant, confusing reactions will occur. An inoculating needle should be used for transferring to the TSI slant, and only the center of the desired colony should be touched. The inoculating needle should then be stabbed into the butt of the slant first and then streaked in a zigzag fashion over the slanted surface. Always remember that a TSI slant should be closed with a cotton plug or a loose closure but never with a tightly fitting cap or stopper.

(2) Figures 5-1 and 5-2 present in a simplified flow-chart form the typical reactions encountered with TSI slants and a general schematic procedure with which to begin classification of the various types of pathogens and potential pathogens. In routine examinations of fecal materials just for enteric pathogens, all TSI slants with acid butts and alkaline slants should be retained for examination; those which are acid throughout (possible E. coli or other coliform bacilli) may be discarded as nonpathogens. The TS1 slants with alkaline slants and neutral (alkaline) butts may also be discarded when dealing with routine fecal specimens. The latter reactions are indicative of Pseudomonas species and Alcaligenes species, which are usually nonpathogens when present in the intestinal tract of adults. Since some Proteus species may be indistinguishable from salmonella and other lactose-nonfermenting gram-negative bacteria on primary isolation, all TSI slant cultures for positive identification should next be screened with urea.

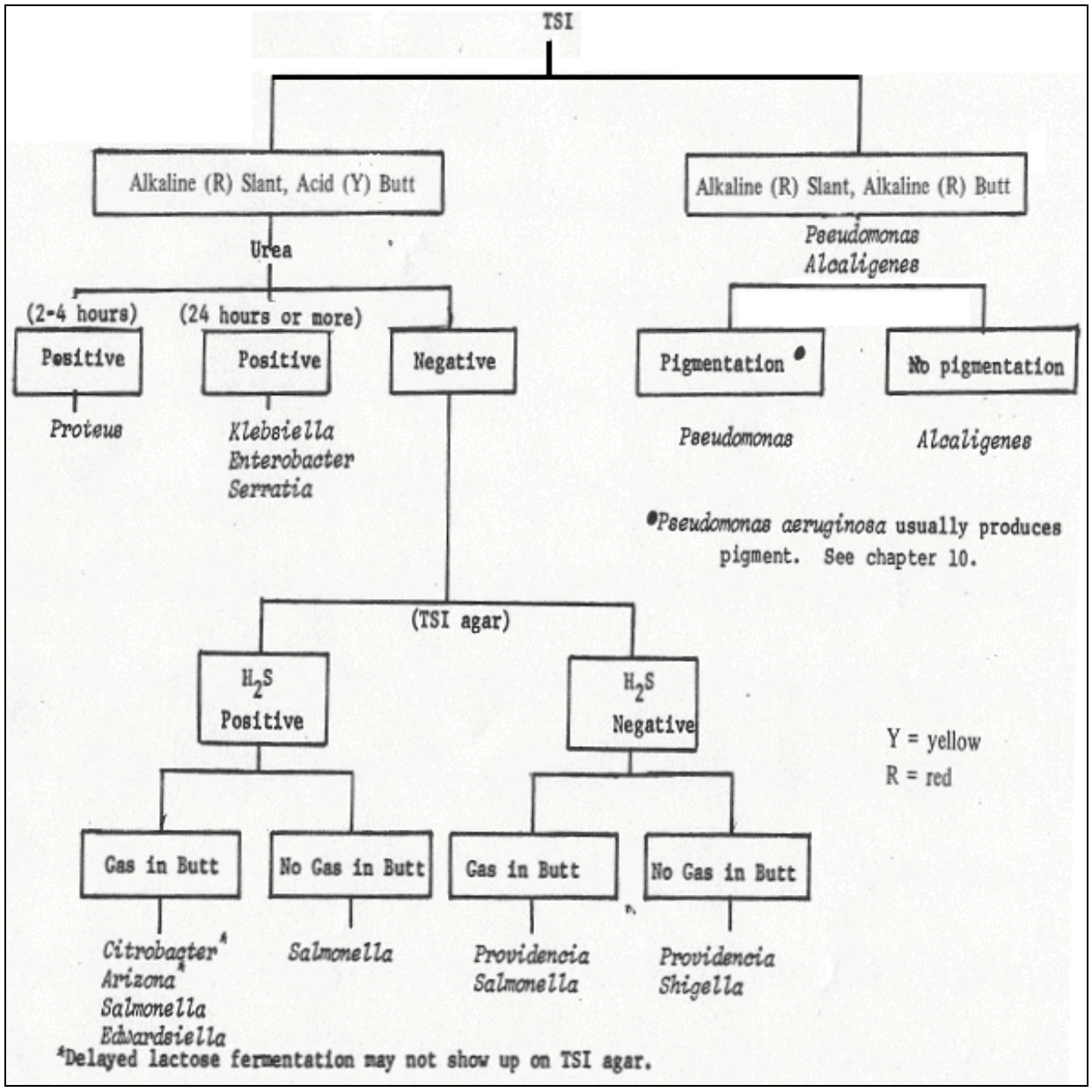


Figure 5-1. Tentative differentiation of lactose-nonfermenting bacilli.

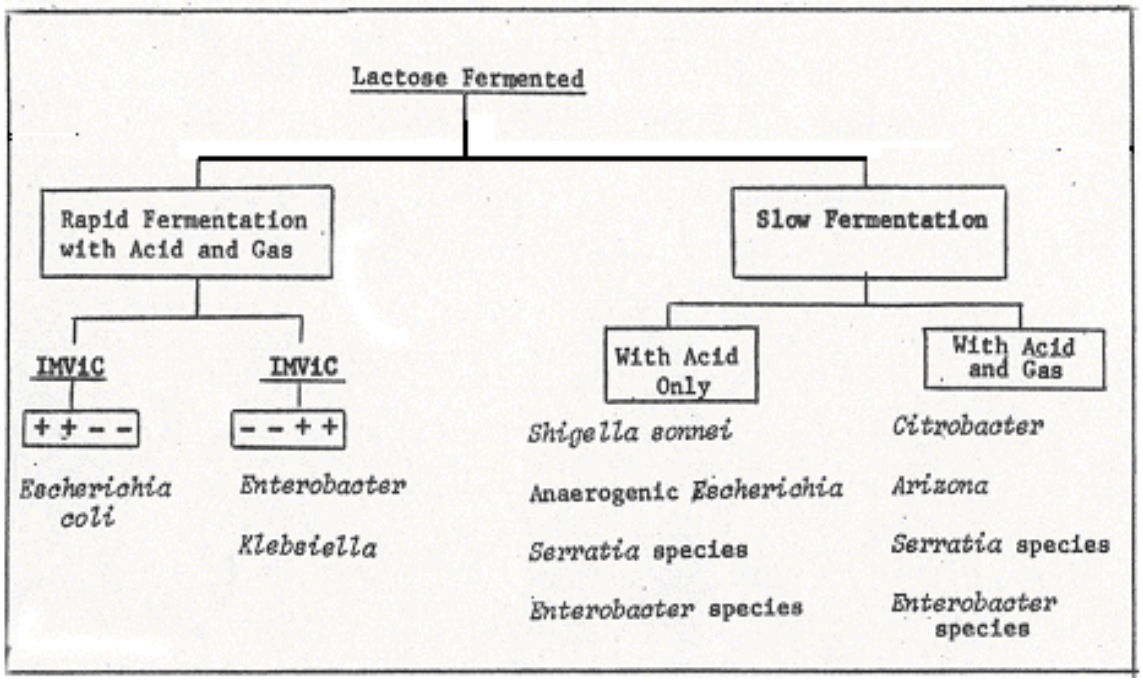


Figure 5-2. Tentative differentiation of lactose-fermenting enteric bacilli.

b. **Urea Media**. Either urea agar slants or urea broth may be used with equal results and in either case a heavy inoculum should be used. Urease activity is detected and observed by a change of color in the indicator system that is due to a pH change caused by the production of ammonia. The splitting of urea to ammonia and carbon dioxide by the enzyme urease may be employed as a reliable test to differentiate Proteus species from other lactose-negative members of the enteric group. Proteus species hydrolyze urea rapidly and release ammonia that is indicated by a reddening of the indicator phenol red. Urease production by the organism under study is evidenced by the medium being converted from light amber to pink. In a negative reaction, no color change takes place. Many organisms cause Christensen's urea agar to turn pink soon after the medium is inoculated. In order for the test results to be valid for Proteus species, the medium must become alkaline (pink color) within the first 2 to 4 hours of incubation.

NOTE: However, hasty conclusions should not be drawn at this point because final identification depends on the results of biochemical and serological confirmation.

5-19. IMVIC TESTS

Most of the members comprising the gram-negative bacilli ferment the carbohydrate lactose although some are very slow fermenters and for this reason will resemble most of the enteric pathogens and must be differentiated from them. The exception to the rule is the pathogen Shigella sonnei, which sometimes is a slow lactose fermenter but seldom causes the production of any gas. The lactose fermenters may be tentatively identified according to figure 5-2 using the IMViC reactions, which are used primarily to differentiate between the coliform bacteria. The letters IMViC stand for the tests indole, methyl red, Voges-Proskauer, and citrate. The lower-case "i" is included only to make IMViC easier to pronounce. See Lesson 3, (paras 3-9 - 3-11) for details about these tests.

a. **Indole Test.** The indole test is based on the ability of certain bacteria to split tryptophan to alanine and indole. The liberated indole will combine with paradimethylaminobenzaldehyde in Kovac's reagent to give a deep red color. The presence of the red color at the interphase between the reagent and the broth culture signifies that indole has been liberated. The absence of the red color signifies a negative test.

b. **Methyl Red Test.** The methyl red test is based on the principle that some organisms ferment glucose, and produce small amounts of acids that are converted to neutral end products. This test is designed to differentiate those organisms that, in contrast, produce high acidity. A positive reaction occurs when the culture is sufficiently acid to turn the methyl red reagent to a distinct red color. A yellow color is regarded as a negative test.

c. **Voges-Proskauer Test.** The Voges-Proskauer test is based on the ability of some organisms to produce a neutral end product, acetylmethylcarbinol, from dextrose. A positive test is indicated by the development of a pink or red color. A yellow color is regarded as a negative test.

d. **Citrate Test.** The citrate test is based on the ability of certain bacteria to utilize sodium citrate when it is the sole available source of carbon in a chemically defined medium. A slant of Simmons' citrate agar is lightly inoculated by streaking only. A positive reaction (growth) is accompanied by an alkaline reaction resulting in a change in the green color of the medium to a deep blue color. No change in the indicator or the absence of blue color in the green medium indicates a negative test. A light inoculum must be used to insure that no nutrients are transferred to the chemically defined medium.

e. **Uses of IMViC Tests.** The IMViC reactions are classically used as a group of tests to separate the Escherichia and the Enterobacter-Klebsiella genera. Escherichia organisms are usually IMViC + + - - and Enterobacter-Klebsiella organisms are usually IMViC - - + +. However, any of these tests are of independent value to aid in the biochemical differentiation of other enteric bacilli.

Section V. IDENTIFICATION OF ENTERIC ORGANISMS

5-20. GENERAL

Figure 5-3 provides an identification schema for the enteric group and related organisms. Table 5-3 describes the reactions of the different enteric organisms to a series of biochemical tests.

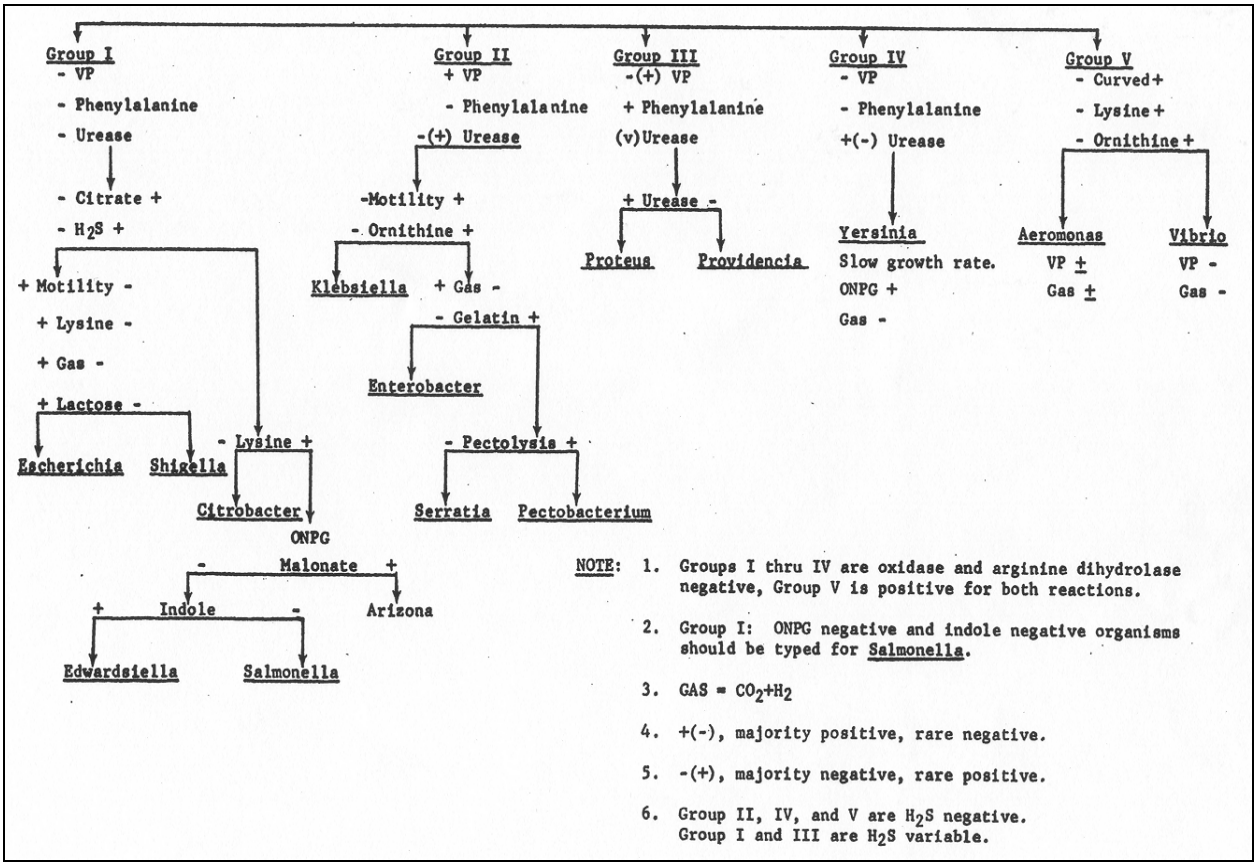


Figure 5-3. Identification schema for the enteric group and related organisms (faculatative gram-positive rods).

TEST or SUBSTRATE	ESCHERICHIEAE		EDWARD-SIELLEAE	SALMONELLEAE			KLEBSIELLEAE						ERWINEAE	PROTEEAE			
	Escherichia	*Shigella*	*Edwardsiella*	*Salmonella*	*Arizona*	*Citrobacter*	*Klebsiella*	*Enterobacter*				*Serratia*	*Pectobacterium*	*Proteus*			*Providencia*
								cloacae	*aerogenes*	*hafniae* 37C	*hafniae* 44C	*liquefaciens* 37C	*liquefaciens* 44C		*vulgaris*	*mirabilis*	*morganii*	*rettgeri*	*alcalifaciens*	*stuartii*
INDOLE	+	-or +	+	-	-	-	- or +	-	-	-	-	-	-	- or +	+	-	+	+	+	+
METHYL RED	+	+	+	+	+	+	-	-	-	+ or -	-	+ or -	- or +	+ or -	+	+	+	+	+	+
VOGES – PROSKAUER	-	-	-	-	-	-	+	+	+	+ or -	+	- or +	+ or -	+	-	- or +	-	-	-	-
SIMMONS'S CITRATE	-	-	-	d	+	+	+	+	+	(+) or -	d	+	+	+	d	+ or (+)	-	+	+	+
HYDROGEN SULFIDE (TSI)	-	-	+	+	+	+ or -	-	-	-	-	-	-	-	-	+	+	-	-	-	-
UREASE	-	-	-	-	-	d[w]	+	+ or -	-	-	-	d	-	d[w]	+	+	+	+	-	-
KCN	-	-	-	-	-	+	+	+	+	+	+	+	+	+ or -	+	+	+	+	+	+
MOTILITY	+ or -	-	+	+	+	+	-	+	+	+	+	d	+	+ or -	+	+	+	+	+	+
GELATIN (22C)	-	-	-	-	(+)	-	-	(+) or -	- or (+)		-	+	+	+ or (+)	+ or (+)	+	-	-	-	-
LYSINE DECARBOXYLASE	d	-	+	+	+	-	+	-	+	+	+	+ or -	+	-	-	-	-	-	-	-
ARGININE DIHYDROLASE	d	- or (+)	-	(+) or +	+ or (+)	d	-	+	-	-	-	-	-	- or +	-	-	-	-	-	-
ORNITHINE DECARBOXYLASE	d	d[1]	+	+	+	d	-	+	+	+	+	+	+	-	-	+	+	-	-	-
PHENYLALANINE DEAMINASE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
MALONATE	-	-	-	-	+	d	+	+ or -	+ or -	+ or -	+ or -	-	-	- or +	-	-	-	-	-	-
GAS FROM GLUCOSE	+	-[1]	+	+	+	+	+	+	+	+	+	+	+	+ or -[3]	+ or -	+	d	- or +	+ or -	-
LACTOSE	+	-[1]	-	-	d	d	+	+	+	+	+	+	(+)	- or (+)	d	-	-	-	-	-
SUCROSE	d	-[1]	-	-	-	d	+	+	+	d	d	+	+	+	+	+	d	-	d	d
MANNITOL	+	+ or -	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+ or -	-	d
DULCITOL	d	d	-	d[2]	-	d	- or +	- or +	-	-	-	-	-	-	-	-	-	-	-	-
SALICIN	d	-	-	-	-	d	+	+ or (+)	+	d	d	+	+	+	+	d	d	-	d	-
ADONITOL	-	-	-	-	-	-	+ or -	- or +	+	-	-	d	d	-	-	-	-	d	+	-
INOSITOL	-	-	-	d	-	-	+	d	+	-	-	+	+	-	-	-	-	+	-	+
SORBITOL	+	d	-	+	+	+	+	+	+	-	-	+	+	-	-	-	-	d	-	d
ARABINOSE	+	d	-	+[2]	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
RAFFINOSE	d	d	-	-	-	d	+	+	+	-	-	+	+	+ or (+)	-	-	-	-	-	-
RHAMNOSE	d	d	-	+	+	+	+	+	+	+	+	-	-	d	-	-	-	+or -	-	-

(1) Certain biotypes of *S. flexneri* produce gas; *S. sonnei* cultures ferment lactose and sucrose slowly and decarboxylate ornithine.
(2) *S. typhi*, *S. choleraesuis*, *S. enteritidis* biotype Paratyphi A and Pullorum, and a few others ordinarily do not ferment dulcitol promptly. *S. choleraesuis* does not ferment arabinose.
(3) Gas volumes produced by cultures of *Serratia*, *Proteus*, and *Providencia* are small.

+, 90 percent or more positive in 1 or 2 days. -, 90 percent or more negative. d, different biochemical types [+, (+) -]. (+), delayed positive. + or -, majority of cultures positive. - or +, majority negative. w, weakly positive reaction.

Table 5-3. Differentiation of Enterobacteriaceae by biochemical tests. (reprinted by permission of Burgess Publishing Company from Identification of Enterobacteriaceae, 1972, 3d Ed., by P.R. Edwards and W.H. Ewing)

5-21. LYSINE DECARBOXYLASE TEST (FALKOW METHOD)

The lysine decarboxylase test measures the enzymatic ability of organism to decarboxylate the amino acid, lysine, causing its conversion into the amine, cadaverine. This activity is characteristic of the genus Arizona, most members of the genus Klebsiella. The test system employs Falkow lysine broth or lysine iron agar, which include glucose, lysine, peptone and the indicator, bromcresol purple (yellow to purple). The initial reaction of the system is acid (yellow, due to the attack on glucose, lysine production of acid end products. Upon depletion of the glucose, lysine is decarboxylated to form the basic compound, cadaverine, which causes reversion of the reaction to alkalinity and the development of a purple color.

5-22. DECARBOXYLASE TESTS (MOELLER METHOD)

a. The basal medium for decarboxylase tests includes a peptone, beef extract, bromcresol purple, cresol red, glucose, and pyridoxal. The basal medium is divided into four portions. One portion, used as a control, is tubed without the addition of an amino acid. To each of the remaining portions, an amino acid is added as follows:

(1) Portion 1--1 percent of L-lysine dihydrochloride

(2) Portion 2--1 percent of L-arginine monohydrochloride

(3) Portion 3--1 percent of L-ornithine dihydrochloride

b. The ph of portion 3 must then be adjusted to 6-6.5 prior to sterilization. Each portion may then be tubed in 3 to 4-ml amounts in small screw-capped tubes and sterilized. After light inoculation of tubes including a control, a 10-mm layer of sterile paraffin is added and the tubes are incubated at 37º C for 4 days. A positive reaction, alkalinization, is indicated when the color changes from a yellow to a violet or reddish-violet color.

5-23. PHENYLALANINE DEAMINASE TEST

The phenylalanine deaminase test has as its basis the ability of the enzyme deaminase in the presence of oxygen to convert the parent compound L-phenylalanine into phenylpyruvic acid. Organisms of the Proteus and Providencia genera are characterized as having such deaminases. This test system requires a culture on L-phenylalanine medium. Phenylpyruvic acid will react with a solution of acidified $Fe_2(SO_4)_3$ and $FeNH_4)_2$ to produce a green color, and it is the development of this green color that makes the phenylalanine deaminase test positive.

5-24. MALONATE TEST

Sterile malonate broth is inoculated from a young agar slant or broth culture; preferably a 3-mm loopful of broth culture. The culture is observed daily during the 48-hour incubation at 37º C. A positive result, utilization of malonate, is indicated by a change of color from green to Prussian blue.

5-25. PECTOLYSIS TEST (MARTIN AND EWING)

a. **Preparation of Medium.** To 100 ml of distilled water is added 0.5 g of yeast extract, 0.9 ml of IN sodium hydroxide, 0.5 ml of 10 percent calcium chloride ($CaCl_2 \cdot 2H_20$), 1.25 ml of 0.2 percent bromthymol blue, 1 g of sodium polypectate No. 24 (Sunkist Growers, Inc., Ontario, California), and 2 g of agar. This is stirred thoroughly, heated gently, sterilized, and poured into thin plates.

b. **Inoculation.** Plates are spot inoculated from a young agar slant. Up to 10 cultures can be tested on a single plate.

c. **Incubation.** Incubate at 37º C for 1 to 3 days.

d. **Interpretation.** Liquefaction of pectate, a positive test, is indicated by depression of the medium around a growth.

5-26. MOTILITY TEST

A tube of motility test medium is inoculated by stabbing to a depth of about 5 mm. It is incubated at 37º C for 1 to 2 days. If the organisms spread out from the line of inoculation, the test is positive. If organisms grow only along the line of the stab, the test is negative. Negative test should be incubated an additional 5 days at 22º to 25º C.

5-27. THE PROTEUS AND PROVIDENCIA GENERA

a. The genus Proteus consists of four species that can be identified by their biochemical patterns. See table 5-3 for typical biochemical reactions of Proteus and Providencia species. Proteus organisms are considered to be normal flora of the intestinal tract, where they usually exist as saprophytes. When grown upon solid media, these bacteria may exhibit the swarming phenomenon and cover the entire plate with a layer of growth. This condition makes recovery of pure cultures of other bacteria present on the plate extremely difficult. Species of Proteus are characterized as being lactose nonfermenters that have the ability to split urea, a characteristic that is extremely helpful in identifying this genus.

(1) Proteus vulgaris and P. mirabilis swarm on a blood agar plate and are usually separated on the basis of indole production; P. vulgaris is indole positive and P. mirabilis is indole negative.

(2) Morganella morganii and P. rettgeri usually do not swarm and are usually separated on the basis of mannitol fermentation and the ability to utilize citrate. Morganella morganii is mannitol positive and citrate positive while Proteus rettgeri is mannitol negative and citrate negative.

(3) Because Proteus species are lactose negative, they are often selected tentatively from isolation plates as colonies of salmonellae or shigellae; Proteus can usually be ruled out by use of the urease test as previously stated.

b. The Providencia species closely resemble Morganella morganii and Proteus rettgeri and for this reason are often included with the genus Proteus. Due to the fact that the Providencia species are H_2S-negative and mayor may not produce gas in glucose, they are often mistaken for shigellae on TSI agar. Members of Providencia may be distinguished, however, from the shigellae by their motility and utilization of citrate, as well as by other biochemical reactions.

5-28. THE ENTEROBACTER AND KLEBSIELLA GENERA

At present it is not easy to distinguish between Klebsiella pneumoniae and Enterobacter aerogenes on a purely biochemical basis (see table 5-3) since their biochemical characteristics are quite similar. However, Klebsiella is comprised of nonmotile organisms and Enterobacter is comprised of motile organisms. Another procedure that is used for differentiation is the ornithine test.

5-29. THE GENUS SALMONELLA

If preliminary biochemical studies are suggestive of a Salmonella species, serological evaluation of the culture is desirable. The serological antigens associated with bacteria are identified by Arabic numerals and alphabetical symbols. The antigens exhibited by the Enterobacteriaceae fall into three main categories--O (somatic), H (flagellar), and K (envelope) antigens.

a. **Somatic Antigens.** Somatic, or body, antigens are assigned the alphabetical symbol O. Somatic antigens are thermostable and determine serological group identification. Using a known specific antiserum that contains antibodies for that antigen identifies each antigen. The reaction of somatic antigens is described as granulation. The time element for this serological reaction is very rapid, occurring within 60 seconds. The granules are very fine and white in color. Any reaction occurring later than 60 seconds should be regarded with suspicion and retested.

b. **Flagellar Antigens.** The flagellar antigens are represented by the alphabetical symbol H and are associated with motility. These antigens are thermolabile and establish species or serotypes within a particular genus or group. H antigens usually do not interfere with or mask the agglutinability of somatic antigens. The reaction of flagellar antigens is described as a flocculation. This reaction is also rapid and occurs within the prescribed 60-second time element. The flocculation is loose and fluffy, appearing a cloudy gray. Most salmonellae are diphasic; that is, each motile type tends to exhibit two antigenic phases, both phases with the same O antigens but different H antigens. To identify the Salmonella serotype, it is necessary to identify the H antigens in both phases.

c. **K ("Capsular") Antigens.** Bacteria may be surrounded by a capsule, sheath, or envelope that possesses K or envelope antigens. The K antigens are thermolabile and assist in further establishing serotypes within a particular genus and species. K agglutination appears fine in texture and white in color. There are varieties or types of K antigens which are designated by the alphabetical symbols L, A, B, and Vi. The K antigens may interfere with O agglutinability and prevent group identification. This reaction is said to mask the somatic antigens, and heat is required to inactivate the K antigens. This makes it possible to test for the O antigens.

d. **Polyvalent Antiserum.** Polyvalent O antisera are available" commercially. Suspected Salmonella species must first be tested with polyvalent antiserum which contains antibodies against Salmonella somatic O antigens A through E. Complete and immediate agglutination is observed with most salmonellae. A few of the paracolons and some shigellae may exhibit partial to complete agglutination due to close similarities of the antigenic structure and the nature of polyvalent antiserum. Those cultures agglutinated by polyvalent antiserum should then be tested with the individual grouping antisera A through E. The particular serum wherein clumping occurs most rapidly is the serological group to which the organism belongs. Although cross reactions between various O groups of the salmonellae do occur, such reactions are usually not pronounced. Any culture that exhibits prompt, definite clumping in polyvalent serum and in one or more of the O grouping sera is reported as a probable Salmonella species. Biochemical confirmation is necessary, for many paracolon bacteria contain antigens related to the O antigens of Salmonella species.

e. **Vi Antiserum.** If a culture fails to react in polyvalent antiserum, it should be tested with Vi ("virulence") antiserum. This antiserum will detect the presence of Vi antigen, which is present on the cell surface of most freshly isolated strains of Salmonella typhi and occasional Salmonella paratyphi C organisms. If agglutination occurs with Vi antiserum, the saline suspension of the organism should be heated in a boiling water bath for about 15 minutes. Upon cooling, the organism is then retested with polyvalent O. Clumping will occur if the unknown is a Salmonella species. Heating removes the Vi (masking) antigen from the cell surface, permitting the O antibodies to react with somatic antigens to produce agglutination. Cultures failing to react with polyvalent or Vi antiserum may be Shigella, Arizona, or Citrobacter species.

(1) Once and agglutination boiled cells should the presence of Vi antigen has been established observed in polyvalent antiserum, the previously be tested with anti-D or anti-C group serum. The particular antisera used are determined by the preliminary biochemical reactions previously observed. Possible Salmonella typhi strains should agglutinate in group D antiserum, and Salmonella paratyphi C cultures should agglutinate in group C antiserum. One should suspect that cultures that react with Vi antiserum and after boiling agglutinate with group D or group C antiserum are, respectively, strains of Salmonella typhi or Salmonella paratyphi C.

(2) All cultures which fail to react quickly and strongly with polyvalent or Vi antiserum should be subjected to further biochemical tests. The same holds true for cultures that react with polyvalent antiserum, but fail to react typically with O grouping antisera. Potassium cyanide medium is excellent in identifying Citrobacter species that can resemble the salmonellae. Citrobacter species grow in KCN broth, while all salmonellae fail to do so.

f. **Species Definition.** Once the group of a Salmonella culture has been determined, species definition is accomplished by subjecting the organism to H (flagellar) antisera, and subsequent biochemical testing. The unheated suspension of the organism is tested on a slide with H antisera (which are diluted 1:50 or 1:100) using the same technique as for O agglutination. Another acceptable technique is to dilute a broth culture of the test organism equally with normal saline containing 0.6 percent formalin and react this with appropriate antisera at 50º C. The antisera for this procedure are diluted 1:100 and the results are read following a one-hour incubation in a water bath. The technique employed will be determined by the specific directions accompanying various lots of antisera procured. It may be necessary on occasion to employ a U-tube or phase-tube to type for both phases of the H antigens. One phase will mask the other preventing complete identification. It is necessary to inoculate the phase tube (which contains a semisolid medium) just below the surface of one end of the tube. Place a small quantity of antisera at the site of inoculation that is specific for the phase you have already identified. The antisera will react with the antigen for which you have established identity and permit the unidentified phase to migrate by means of motility to the opposite end of the tube. Remove the motile bacteria that have reached this point and inoculate to an agar slant and subsequently complete typing for the flagellar antigens of the organism in question. Most bacteria of the Salmonella genus possess both phase 1 and phase 2 antigens. If an organism has two phases of H antigens, you must establish the complete flagellar typing for both phases.

g. **Reactions of Salmonella typhi.** The typhoid bacillus is one of the most frequently encountered Salmonella species. It is important that the typhoid bacillus be recognized as a highly specialized member of the genus Salmonella with certain characteristics that set it apart from most of the other salmonellae. Salmonella typhi produces acid in the typical media used for biochemical studies, but it does not produce gas as do most members of this genus. Salmonella typhi produces an acid reaction without the production of gas in the fermentation of glucose, mannitol, trehalose, maltose, and dextrin. It does not ferment lactose or sucrose; no gas is formed in a TSI slant and the production of H_2S on TSI is variable. Indole is not produced and gelatin is not liquefied.

5-30. THE GENUS SHIGELLA

a. **Antiserum Reactions.** Stool cultures suspected of being Shigella should be tested with the individual polyvalent Shigella grouping antisera. Four antisera are usually recommended for Shigella species, each containing antibodies specific for members of the four Shigella subgroups--subgroup A (S. dysenteriae), B (S. flexneri), C (S. boydii), and D (S. sonnei). Specific agglutination usually takes place within one minute with most commercially prepared antisera. The saline and normal serum controls should reveal no clumping. Delayed or incomplete cross-reactions may be observed between certain members of the Shigella subgroups, but only the prompt or complete reactions are significant. Such cross-reactions are due to antigenic similarities between various Shigella subgroups. It is important to remember that other Enterobacteriaceae are occasionally encountered which possess somatic O antigens related to those of the genus Shigella. For this reason, cultural and biochemical studies, as well as serological procedures, should be employed for identification of suspected cultures.

b. **Extended Testing.** If an organism appears to be a Shigella species yet fails to agglutinate in any of the antisera, a saline suspension should be heated in a boiling water bath for 30 to 60 minutes and then retested. Many Shigella cultures possess envelope surface antigens (K antigens) that prevent the somatic antisera from coming in contact with somatic antigens. Therefore, specific agglutination is impossible unless the surface antigen is first inactivated by heat. When a suspected Shigella culture still fails to react with Shigella grouping sera even after heating, the unheated organism should be tested with Salmonella polyvalent and Vi antisera. This is necessary, because certain nonmotile Salmonella typhi strains which fail to produce hydrogen sulfide (18 to 24 hours) resemble the shigellae on triple sugar iron agar slants. Other atypical salmonellae are occasionally encountered which resemble Shigella species upon tentative biochemical and cultural analyses.

c. **Characteristics of Shigella.** All species of Shigella are nonmotile. Other characteristics of the genus Shigella are as follows: All species ferment glucose with no formation of gas (the exception is S. flexneri, which may be aerogenic); no species produce gas on any of the other carbohydrate media; some ferment mannitol and others do not; there is no fermentation of salicin or adonitol; they do not grow on Simmons' citrate agar; and they do not hydrolyze urea, liquefy gelatin, or form acetylmethylcarbinol. All strains of Shigella are lactose nonfermenters with the exception of S. sonnei that is a very delayed lactose fermenter (4 to 7 days).

5-31. THE GENUS ESCHERICHIA

The genus Escherichia is often not thought of as including those organisms formerly referred to as the Alkalescens-Dispar group. The Alkalescens-Dispar strains closely resemble other Escherichia organisms in biochemical reactions and serologic complexity; however, they neither ferment lactose nor produce gas from other carbohydrates. The typical Escherichia colonies are easily recognized and are characterized by their rapid fermentation of lactose with gas formation, and by the classic IMViC reactions. The Alkalescens-Dispar strains are now considered as separate serotypes of E. coli.

a. **Antigenic Tests.** The antigenic structure of the escherichiae is almost as complex as that of the salmonellae. Three types of antigens possessed by E. coli strains are important in their serological identification. These are O (somatic, cellular) antigens that resist heat inactivation at 100º C for one hour, the H (flagellar) antigens that are inactivated by exposure to heat at 100º C for one hour, and the K (enveloping, capsular) antigens which surround the O antigens and prevent their agglutination in specific O antiserum. The K antigens are inactivated by exposing the organisms to heat at 100º C for one hour.

b. **Serotype Identification.** Isolation of E. coli from infant diarrhea requires that the fecal material be inoculated to differential media (EMB, MacConkey's or deoxycholate agar) rather than the more highly selective media used for isolation of Salmonella and Shigella species; otherwise, growth will be inhibited. Blood agar should also be inoculated since occasional E. coli strains fail to develop on differential media. After 18 to 24 hours' incubation the plates are examined for typical colonies of E. coli.

(1) Since the colonies of enteropathogenic forms cannot be grossly distinguished with certainty from nonpathogenic forms, several colonies should be tested serologically. This may be quickly and effectively accomplished by drawing an inoculating loop across a number of colonies and preparing an emulsion of the growth in a drop of saline on a glass slide. A loopful of pooled (polyvalent) antiserum is mixed with the suspension. The antiserum pool contains specific antibodies against each of the OB antigens of the eleven E. coli serotypes most often associated with infant diarrhea. Pronounced agglutination observed in the organism-antiserum mixture is presumptive evidence for the presence of an enteropathogenic E.coli serotype.

(2) Following such results individual colonies are confirmed in the pools and subsequently tested with individual sera to identify the specific type. To accomplish this, depending upon the amount present on the primary plates, it may be necessary to subculture growth on a nutrient agar slant before preparing suspensions for serotyping. Agglutination of a given serotype occurs rapidly in its specific antiserum.

5-32. MORPHOLOGY

Yersinia are reasonably large (0.5 to 1.0 by 1 to 2 microns) gram-negative coccobacilli that are ovoid or rod-shaped. Yersinia (Pasteurella) pestis is encapsulated, nonmotile, nonsporogenous, and characteristically bipolar staining. Other species are Yersinia enterocolitica and Yersinia pseudotuberculosis that are not discussed further in this study guide.

5-33. CULTURAL CHARACTERISTICS

Y. pestis is not fastidious with respect to nutritive requirements, although trypticase soy agar and broth are recommended for its primary cultivation and blood agar produces the best growth. Once isolated, the organism may be subcultured on ordinary media. After 48 hours' incubation on trypticase soy blood agar, Y. pestis will appear as small (0.1 to 0.2 mm), round, glistening, transparent, colorless colonies that are droplet-like and nonhemolytic. Unlike most bacteria that are pathogenic for man, Y. pestis grows best at 25º to 30º C; however, 37º C is acceptable for incubation. Broth cultures appear turbid or contain flocculent growth. Older colonies become larger, more opaque, and develop grayish-yellow centers with gray-white edges.

5-34. LABORATORY IDENTIFICATION

a. **Culture.** When plague is suspected, materials aspirated from buboes and sputum should be examined. Blood specimens should also be cultured early and late in the course of infection when fever is high. At autopsy, materials from lesions and inflammatory areas of internal organs, especially the spleen, will yield the organisms. Y. pestis may be isolated from blood using routine methods. For specimens other than blood, Trypticase soy (blood agar and broth) media are employed for Y. pestis. When plague is suspected, it is imperative that special media be inoculated in addition to the routine media (blood agar and thioglycollate broth). This is necessary since other bacterial agents may cause systemic disease similar to plague.

CAUTION: The plague organisms are extremely dangerous pathogens, and strict aseptic technique must be maintained at all times.

b. **Observable Details.** Solid media are examined for typical colonies suggestive of Y. pestis. Smears should be prepared of these as well as any growth present in broth media. The tendencies for bipolar staining is demonstrated well with simple methylene blue, crystal violet, or dilute carbolfuchsin stain. Y. pestis reduces nitrites but fails to produce indole, and it is catalase positive and urease negative. It produces scant growth of reddish colonies when subcultured on a plate of deoxycholate citrate agar.

c. **Definitive Identification.** Definitive identification involves the use of bacteriophages (viruses), an agglutination test, a fluoresecent-antibody test, and animal inoculation. Reagents for the first three of these are not commercially available. Animal inoculation should be undertaken only where special facilities are available.

5-35. PATHOGENICITY

Yersinia pestis is the etiological agent of plague, a disease primarily of rodents which is secondarily transmitted to man.

a. One of the more commonly affected rodents is the wild rat, but guinea pigs, squirrels, prairie dogs, and mice are also susceptible. Plague is spread among rodents through the bites of fleas, previously infected via a blood meal from an infected animal. Human plague results when an infected flea feeds on man.

b. Following entry of the plague bacilli into the body, the organisms spread by way of the lymph channels to the regional lymph nodes. The lymphatic channels and nodes become inflamed, hemorrhagic, and greatly enlarged, forming buboes that are usually located in the groin or axilla. Such infections are referred to as bubonic plague, and milder forms of the disease are more or less restricted to the lymphatic system. In many cases, the organisms spread to the blood stream and are distributed to all organs, particularly the spleen, liver, and lungs. The parenchymatous tissues become inflamed and hemorrhagic, ultimately leading to local necrosis. Death may result from a meningitis or overwhelming septicemia.

c. The septicemia phase is sometimes accompanied by subcutaneous hemorrhages that cause the formation of dark spots on the skin. Because of this, the plague is sometimes called the "black death".

d. Since bubonic infections may progress to involve the lungs, individuals so affected disseminate plague to the respiratory tracts of other persons by coughing or sneezing, producing a highly infectious aerosol. Primary pulmonary infections (pneumonic plague) are always fatal when untreated.

Continue with Exercises

EXERCISES, LESSON 5

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. Motile organisms belonging to the family Enterobacteriaceae are:

   a. Atrichous.

   b. Monotrichous.

   c. Amphitrichous.

   d. Lophotrichous.

   e. Peritrichous.

2. Escherichia coli is a gram-:

   a. Positive coccus.

   b. Negative coccus.

   c. Positive bacillus.

   d. Negative bacillus.

3. Escherichia coli is:

   a. ALL OF THE BELOW.

   b. Part of the normal flora of the intestinal tract.

   c. An organism sometimes associated with infant diarrhea.

   d. A frequent cause of urinary tract infections.

4. On TSI agar, organisms responsible for typhoid fever and bacillary dysentery usually produce the following results:

    a. Red slant, red butt.

    b. Yellow slant, red butt.

    c. Red slant, yellow butt.

    d. Yellow slant, yellow butt.

5. The genus Shigella includes the:

    a. Paratyphoid bacilli.

    b. Aberrant coliforms.

    c. Typhoid bacilli.

    d. Dysentery bacilli.

6. Most of the enteric pathogens fall into the category of being:

    a. Lactose fermenters.

    b. Lactose nonfermenters.

    c. Late lactose fermenters.

    d. Able to ferment lactose with the formation of gas.

7. The "coliforms" are generally categorized as being:

    a. Late lactose fermenters.

    b. Lactose nonfermenters.

    c. Unable to grow on a medium containing lactose.

    d. Able to ferment lactose with the formation of gas in 24 hours.

8. Colonies of Escherichia coli have a green metallic sheen on which agar?

   a. SS.

   b. MacConkey's.

   c. EMB.

   d. Deoxycholate.

9. MacConkey's agar is a differential medium because:

   a. Only Salmonella and Shigella will grow on it.

   b. Gram-positive organisms are inhibited from growing on it.

   c. Lactose fermenters may be distinguished from nonfermenters when they grow on it.

   d. No gram-negative rods are able to grow on it.

10. If an organism ferments lactose, it will produce pink or red colonies on Salmonella Shigella agar.

    a. True,

    b. False.

11. Which of the following media should NOT be autoclaved?

    a. EMB agar.

    b. MacConkey's agar.

    c. Bismuth sulfite agar.

    d. Brilliant green agar.

12. Tetrathionate broth is especially valuable for promoting the recovery of:

    a. Shigella.

    b. Salmonella.

    c. Coliforms.

    d. Gram-positive bacteria.

13. Triple sugar iron agar includes which of the following sugars?

    a. Lactose, glucose, and sucrose.

    b. Maltose, lactose, and sucrose.

    c. Lactose and sucrose only.

    d. Lactose and glucose only.

14. Reactions on TSI agar cannot be properly interpreted if the slants are incubated more than 48 hours. These reactions should ideally be observed after an incubation of how long?

    a. 10 to 16 hours.

    b. 18 to 24 hours.

    c. 26 to 32 hours.

    d. 34 to 40 hours.

15. In the butt of a TSI tube, gas production is evidenced by:

    a. Bubbles.

    b. A pink color.

    c. A black color.

    d. A yellow color.

16. In the isolation of enteric pathogens from fecal specimens, what type of medium is particularly recommended if Salmonella typhi is suspected?

    a. EMB agar.

    b. Deoxycholate agar.

    c. MacConkey's agar.

    d. Bismuth sulfite agar.

17. Salmonella species produce a pink-white colony surrounded by a brilliant red zone on which of the following types of agar media?

    a. Bismuth sulfite agar.

    b. SS agar.

    c. Brilliant green agar.

    d. EMB agar.

18. A TSI slant culture after the usual period of incubation at 37^{O} C shows an alkaline slant, an alkaline butt and growth with a slightly greenish pigmentation will most likely prove to be which of the following?

    a. Salmonella.

    b. Shigella.

    c. Pseudomonas.

    d. Proteus.

19. Which of the following may be employed as a reliable test to differentiate Proteus,species from other lactose negative enterics?

    a. Urea broth.

    b. The "swarming" factor of Proteus.

    c. TSI agar slant.

    d. Selenite F broth.

20. A TSI slant culture develops an acid butt and an alkaline slant after the usual period of incubation at 37º C. When grown on a urea medium, the same bacterial strain produces a positive reaction (production of urease) within 2 to 4 hours. The bacterial strain belongs to what genus?

    a. Klebsiella.

    b. Salmonella.

    c. Providencia.

    d. Proteus.

21. The typical IMVIC reaction for the Enterobacter-Klebsiella genera is:

    a. - - + +.

    b. + + - -.

    c. + - - +.

    d. - + + -.

22. The IMVIC reactions of Escherichia coli are:

    a. - - + +.

    b. + - + -.

    c. + + - -.

    d. + - - +.

23. Proteus vulgaris and Proteus mirabilis both swarm on blood agar and are separated on the basis of:

    a. Methyl red.

    b. Indole production.

    c. Citrate.

    d. Motility.

24. To distinguish between Proteus mirabilis and Salmonella species, which of the following would be most useful?

    a. Color of the butt of the triple sugar iron agar.

    b. Color of the colonies on MacConkey's agar.

    c. Reaction in the urease test medium.

    d. Growth in semisolid motility medium.

25. What isolate should have the following characteristics?

    Aerobic gram-negative bacilli.
    T51 agar--no change in the slant, yellow butt with gas, no blackening of the medium.
    Motile urease test medium--no change, growth in KCN medium.
    Growth on phenylalanine agar turns green when ferric chloride is added.

    a. Escherichia.

    b. Providencia.

    c. Pseudomonas.

    d. Klebsiella.

26. What isolate should have the following characteristics?

Aerobic gram-negative bacilli.
On MacConkey's agar--large, slimy, spreading, pink-white colonies. on
TSI agar--yellow throughout with gas in the butt, no blackening of the medium.
Nonmotile.
Urease test medium-positive reaction.
Citrate test medium-positive reaction

a. Providencia.

b. Neisseria.

c. Bacillus.

d. Klebsiella.

27. A facultative gram-negative rod gives the following biochemical reactions: VP positive, phenylalanine negative, motility negative, and oxidase negative. This organism could be:

a. Vibrio cholerae.

b. Yersinia pestis.

c. Klebsiella pneumoniae.

d. Shigella dysenteriae.

e. Proteus rettgeri.

28. Which of the following types of antigens is heat stable?

a. Somatic.

b. Flagellar

c. Envelope.

d. Virulence.

29. In order to identify a Salmonella serotype, it is necessary to identify the _____ antigens in both phases.

    a. Somatic.

    b. Flagellar.

    c. Envelope.

    d. Capsular.

30. If a suspected Shigella culture fails to agglutinate with polyvalent "0" antiserum, the culture should be heated to inactivate a possible _____ antigen that may be masking the somatic antigens.

    a. H.

    b. C.

    c. K.

    d. Vi.

31. Shigella can be differentiated from Salmonella because Shigella organisms are normally:

    a. Citrate positive and motile.

    b. Citrate negative and nonmotile.

    c. Glucose positive with acid and gas production.

    d. Lactose negative.

32. Pathogenic strains of E. coli may be differentiated from nonpathogenic strains on the basis of:

    a. Biochemical reactions.

    b. Serological typing.

    c. Morphology.

    d. Gram reaction.

33. Yersinia pestis, when stained, characteristically shows:

    a. Gram-positive bacilli.

    b. Bipolar staining.

    c. Metachromatic granules.

    d. Gram-negative cocci.

34. Which staining phenomenon characterizes the Yersinia organisms?

    a. ALL OF THE BELOW.

    b. Bipolar staining.

    c. Acid-fast staining.

    d. Metachromatic granules.

35. Plague, known as the "black death" because of the areas of skin darkened by subcutaneous hemorrhages, is caused by:

    a. Shigella dysenteriae.

    b. Salmonella typhi.

    c. Klebsiella pneumoniae.

    d. Yersinia pestis.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES, LESSON 5

1. e (para 5-1)
2. d (para 5-1)
3. a (par 5-4)
4. c (paras 5-8a; 5-9; table 5-1)
5. d (para 5-9)
6. b (para 5-11c)
7. d (para 5-11a)
8. c (para 5-12a; table 5-2)
9. c (para 5-12b)
10. a (para 5-13b)
11. c (para 5-13c)
12. b (para 5-14b)
13. a (para 5-15a(2))
14. b (para 5-15b(3))
15. a (para 5-15b(3)(d))
16. d (para 5-16)
17. c (table 5-2)
18. c (para 5-18a(2); table 5-1; fig 5-1)
19. a (para 5-18b)
20. d (para 5-18b; fig 5-1)
21. a (para 5-19e; fig 5-2)
22. c (para 5-19e; fig 5-2; table 5-3)

23. b (para 5-27a(1); table 5-3)

24. c (para 5-27a(3))

25. b (paras 5-27a, 5-23; table 5-3; fig. 5-1; 5-3)

26. d (para 5-28; tables 5-1, 5-2, 5-3; fig 5-2, 5-3)

27. c (para 5-28; table 5-3; fig 5-3)

28. a (para 5-29a)

29. b (para 5-29b)

30. c (para 5-30b)

31. b (para 5-30c; table 5-3)

32. b (para 5-31b(1))

33. b (para 5-32)

34. b (para 5-32)

35. d (para 5-35)

End of Lesson 5

LESSON ASSIGNMENT

LESSON 6	Other Pathogenic Gram-Negative Bacilli and Antibiotic Sensitivity Tests.
LESSON ASSIGNMENT	Paragraph 6-1 through 6-23.
LESSON OBJECTIVES	Upon completion of this lesson, you should be able to:

6-1. Select descriptive characteristics of the procedures for identifying oxidative and nonreactive bacteria, with emphasis on Pseudomonas.

6-2. Identify descriptive features of Brucella, Haemophilus Bordetella, Francisella tularensis, Bacteroides fragilis, and Streptobacillus moniliformis and the procedures for identifying the species.

6-3. Identify the purpose and principles of antibiotic sensitivity tests and characteristics of the paper disk method and tube dilution method.

6-4. Given a Kirby-Bauer zone size, the name of an antibiotic, and type of organism, indicate whether the organism is resistant, sensitive, or intermediate in sensitivity.

SUGGESTION After reading and studying the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 6

OTHER PATHOGENIC GRAM-NEGATIVE BACILLI AND ANTIBIOTIC SENSITIVITY TESTS

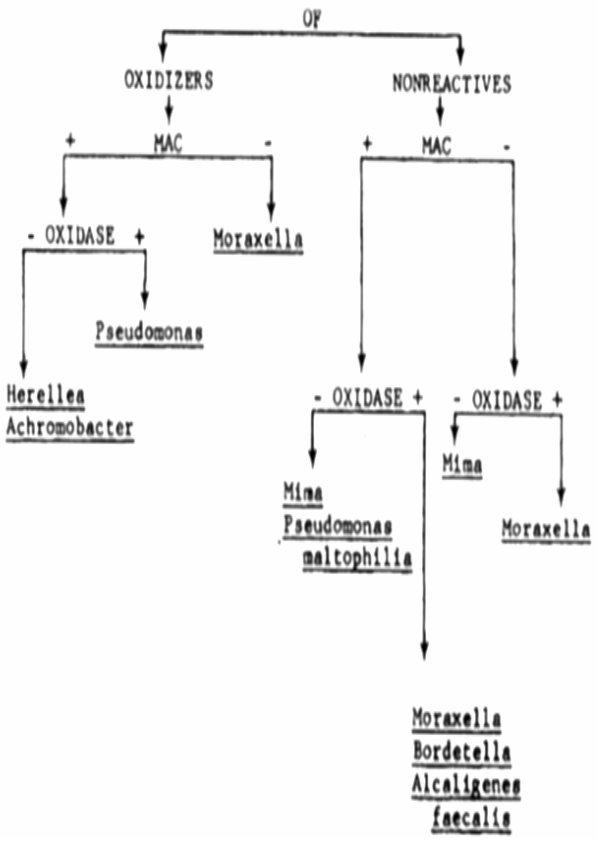
Section I. PSEUDOMONAS

6-1. INTRODUCTION

a. General information.

(1) Figure 6-1 provides an identification schema for oxidative and nonreactive gram-negative bacteria.

ORGANISM	OXIDASE	OF (Glucose)	NO_3 to NO_2	MacC	Motility
Pseudomonas	+ (-)	O	-	+	+ (-)
Achromobacter	-	O	+, -	+, -	+ (-)
Herellea	-	O	-	+	-
Flavobacterium	+, -	O, N	-	+, -	+ (-)
Alcaligenes	+	N	+	+	+
Mima	-	N	-	+, -	-
Moraxella	+	N	-	+, -	-
Bordetella bronchiseptica	+	N	+	+	+



O = Oxidate
N = Nonreactive
+ (–) = Positive, rear negative
MAC = MacConkey's agar (+ = growth, = no growth)

Figure 6-1. Identification schema for oxidative and nonreactive gram-negative bacteria.

(2) Figure 6-2 provides an identification schema for Pseudomonas species in particular.

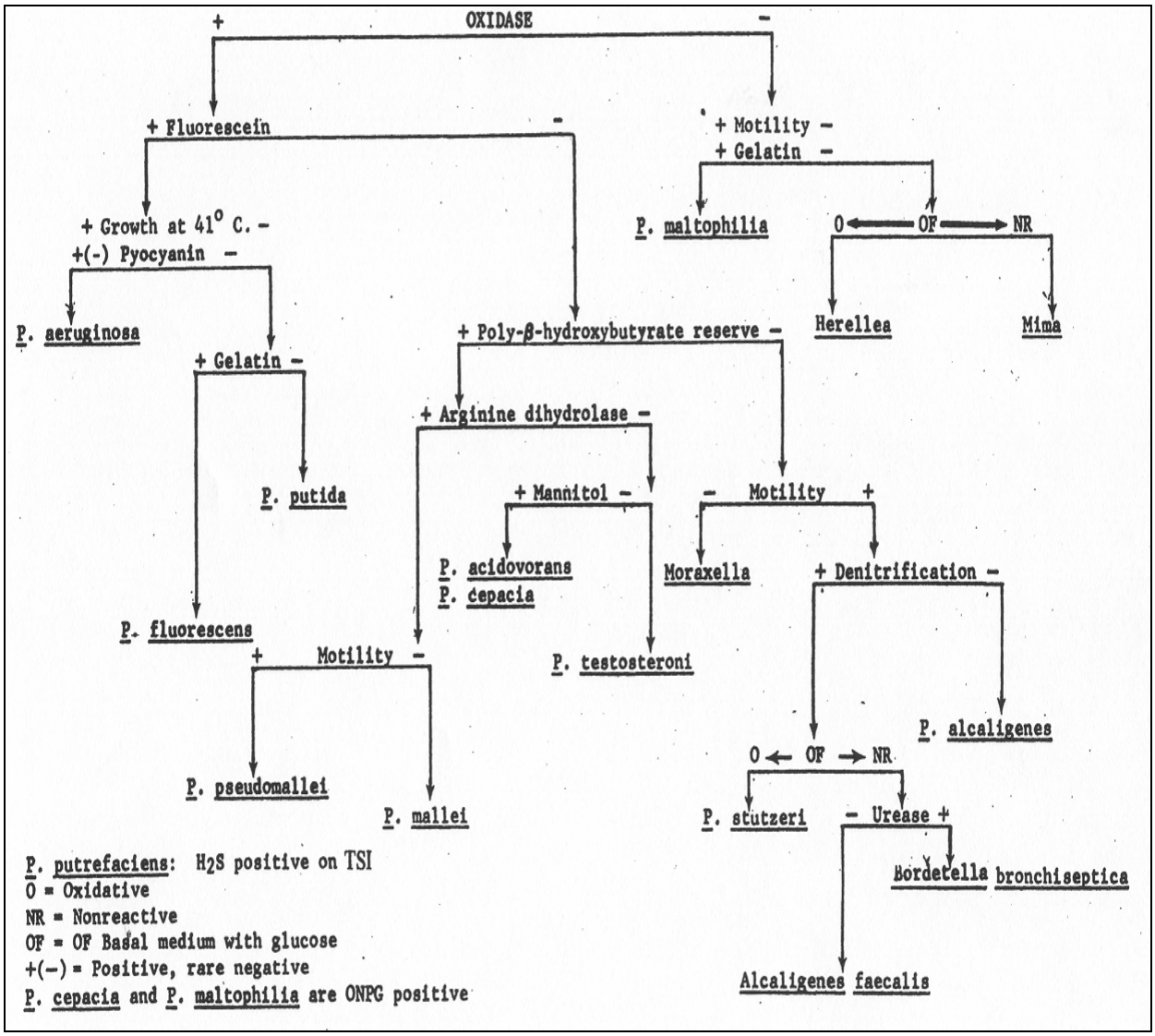


Figure 6-2. Identification schema for Pseudomonas species (TSI): no change)

(3) Table 6-1 indicates the reactions of the different Pseudomonas species to a selected group of tests

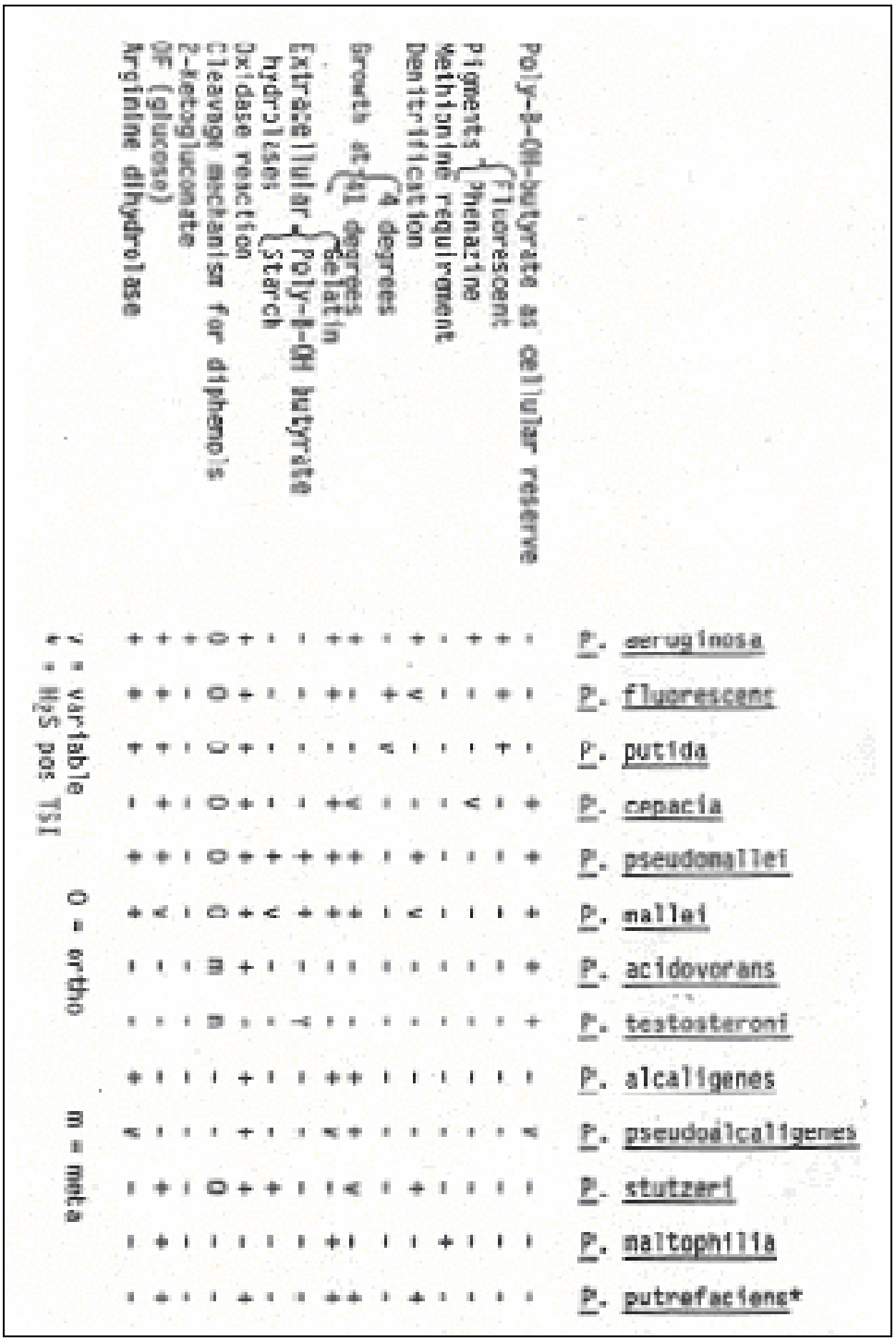


Table 6-1. General characters of diagnostic value for the differentiation of the genus Pseudomonas.

b. Pseudomonads are gram-negative, asporogenous, catalase-producing rods commonly found in soil and water. Indole, methyl red, and Voges-Proskauer tests are negative. Most species are motile, with one polar flagellum or several polar flagella; some are nonmotile and atrichous. Unlike the Enterobacteriaceae, which only ferment carbohydrates, the pseudomonads oxidize carbohydrates and do not obtain energy by fermentative or photosynthetic metabolism.

c. A useful medium called oxidative-fermentative (OF) basal medium has been especially designed to help differentiate between carbohydrate-oxidizing organisms and those that only ferment carbohydrates.

(1) Unlike most fermentation media, basal medium contains about 0.2 percent peptone rather than 1 percent peptone. The higher concentration tends to result in enough alkalinity, as the peptone is attacked, to mask the acidity produced by oxidative organisms. OF basal medium, which contains 1 percent carbohydrate, changes color from blue-green to yellow as acid is produced.

(2) For each isolate, each of two tubes is inoculated by stabbing once with a straight needle, almost to the bottom of the agar. One of the two tubes is layered with sterile mineral oil or melted petroleum.

(3) During incubation, oxidizers produce acidity in the open tube but not the covered tube. Fermentative organisms generally produce acidity in both tubes. Nonreactive organisms fail to produce acidity in either tube. If the organism has grown only along the stab line, it is nonmotile. If it has grown out from the stab line, it is motile.

6-2. PSEUDOMONAS AERUGINOSA

a. **Pathogenicity.** Pseudomonas aeruginosa may infect surgical wounds, severe burns, and other injuries, where it tends to produce a characteristic blue-green pus. Since it is resistant to antibiotic therapy, it tends to produce dangerous infections at the sites of previous infections eradicated by antibiotics. Infections of the eye, the ear, and the urinary tract are frequently reported. Systemic infections, which may occur in individuals with lowered resistance, tend to be fatal.

b. **Identification.** P. aeruginosa is most easily identified by its characteristic blue-green pigment, but 4 percent of its strains do not produce this coloration. On agar the colonies are large, flat, with a ground glass appearance, and they tend to spread, especially on nutrient agar or Trypticase soy agar. They have a characteristic grapelike odor. If an organism meets the following criteria, it may be identified as P. aeruginosa:

(1) Polar monotrichous (generally having a single polar flagellum).

(2) Gram-negative, asporogenous, rod-like.

(3) Oxidation of glucose.

(4) Failure to oxidize maltose.

(5) Production of indophenol oxidase.

(6) Reduction of nitrate to gas.

(7) Oxidation of gluconate to ketogluconate.

(8) Growth at 42º C. (Most strains will grow form 25º to 42º C; some will grow from 5º to 10º C.)

6-3. PSEUDOMONAS PSEUDOMALLEI

a. **Introduction.** Melioidosis is an infectious disease caused by a gram-negative, motile bacillus, Pseudomonas pseudomallei, which is found in soil and water in Southeast Asia. The disease is manifested in a number of ways ranging from an inapparent infection to a fatal septicemia. However, the most common manifestation is an acute pneumonia or pneumonitis. The mode of transmission has not been definitely established; however, there are several possible routes. Pseudomonas pseudomallei has been isolated from soil, local fruits and vegetables, well water, and variety of surface waters. Attempts to identify a human or animal reservoir have proven unsuccessful to date. Cultures have been performed on the urine and feces of humans, rats, goats, cattle and chickens. Thus far, on the basis of these cultures, no human or animal reservoir has been identified and it is assumed that the organism leads a saprophytic existence in nature. Likewise, there has been no proof of man-to-man transmission. The disease is frequently associated with traumatic injuries.

b. **Identification.** Early diagnosis of melioidosis should be considered in all cases of febrile disease in persons in an endemic area and in those recently returned from such an area. The only positive laboratory identification of melioidosis is the isolation and identification of Pseudomonas pseudomallei. It is isolated from the sputum in the pneumonic form. In other cases blood, urine, feces, spinal fluid, and surgically removed tissue have yielded positive cultures. Culturally the organism grows well in 2 to 3 days on ordinary media such as Trypticase soy agar and blood agar. It is aerobic in its oxygen requirements. Colonies on agar medium appear circular, raised, opaque, creamy, and yellow to brown in color with irregular edges. The colonies tend to become wrinkled after several days (four to five), and this is one of the diagnostic characteristics of the organism. Due to this phenomenon it is important that cultures in all suspected cases be held so that this may be observed. Cultures of the organism give off an earthy (ammoniacal) odor. Gelatin slabs show moderate, crateriform liquefaction. Litmus milk is curdled with slow acid production. The following commonly employed biochemicals are oxidized with the production of acid but no gas: glucose, maltose, lactose, mannitol, and cellobiose. Due to the unfamiliarity of many bacteriologists with the organism, it is sometimes confused with other gram-negative bacilli and has probably been reported as Escherichia coli, Pseudomonas aeruginosa, or one of the Klebsiella or Enterobacter species in a number of cases.

Section II. BRUCELLA

6-4. GENERAL TYPES

The genus Brucella includes several species known to infect man. However, man is not the primary host of any of these. Goats are infected by B. melitensis, cattle by B. abortus, swine by B. suis, and dogs by B. canis. Man can be infected with any of these organisms by direct contact or by consumption of milk or milk products. The brucellae exhibit predominantly small coccobacillary forms ranging from 0.4 to 3.0 microns in length and 0.4 to 0.8 microns in breadth. Fresh isolates from disease are encapsulated and form smooth mucoid colonies on agar media. The cells occur singly, in pairs, or in short chains. Brucella species do not possess flagella, nor do they form spores.

6-5. CULTURE

a. The brucellae require complex media for growth. Although many special media have been devised and recommended for cultivation of Brucella species, Trypticase soy broth and agar are used with considerable success.

b. Brucella abortus can be cultivated from clinical specimens only under increased CO_2 tension, preferably 10 percent. Brucella suis and Brucella melitensis will grow with or without increased carbon dioxide but their growth is enhanced by increased CO_2 tension.

c. Prolonged incubation (several weeks) at 37° C is often necessary for initial isolation of Brucella species; however, upon subculture, growth usually occurs within 3 or 4 days. Colonies of Brucella species are small, translucent, smooth, glistening, and blue-gray in color, and they possess entire (even) margins.

d. It is necessary to keep the culture 21 days before reporting a negative result.

6-6. PATHOGENICITY

Brucella abortus, Br. melitensis, Br. suis, and Br. canis are all pathogenic for man. Following the ingestion of raw milk from infected animals, the brucellae may invade the oral mucous membranes or the lining of the alimentary tract. Infections also result from direct contact with infected tissue. Occasional cases of pulmonary brucellosis suggest that infections may be acquired by inhalation. The incidence of brucellosis is much higher among slaughterhouse attendants, veterinarians, sausage-makers, butchers, dairymen, or similar occupational groups exposed to infected animals.

a. After entry into the human host, the organisms progress by way of lymphatic channels to the thoracic duct. They enter the blood stream and are widely disseminated

to various tissues including the liver, spleen, bone marrow, and other areas of the reticuloendothelial system. The organisms form multiple intracellular abscesses in the particular tissue affected. Osteomyelitis or meningitis may occur.

b. Characteristic symptoms begin insidiously (usually 10 to 14 days following infection), with slight periodic fever, weakness, and malaise. The lymph nodes and spleen gradually become enlarged; deep pain and symptoms of in coordination may also occur. At the height of infection, acute febrile episodes occur as a result of organisms being periodically released into the blood stream. Frank symptoms usually subside within three months and the bacilli remain dormant in deep tissues (chronic brucellosis) and long as the general physiological well being of the individual is maintained. Relapses of acute symptoms may occur when the resistance of the infected individual is lowered.

6-7. LABORATORY IDENTIFICATION

a. **Isolation.** The specimens usually examined for Brucella species are blood samples taken during the febrile stage. Lymph aspirations, biopsy materials, spinal fluid, or swab specimens of deep lesions may also be examined. All specimens from suspected brucellosis should be inoculated on Trypticase soy agar and broth. Blood specimens should be collected in a Castaneda bottle if they are available. All specimens must be cultivated at 37º C under 10 percent carbon dioxide. Cultures should be held at least 30 days before being discarded as negative. Although the gram-negative, coccobacillary cells of Brucella species may be observed in sputum or deep tissue fluids, such smears are rarely of diagnostic value since other bacteria may exhibit similar morpholgy

CAUTION: The brucellae are dangerous pathogens and strict aseptic technique must be maintained at all times. A bacteriological hood should be employed.

b. **Differentiation.** To differentiate the species many procedures are available. Agglutinin absorption will separate Br. melitensis from Br. suis and Br. abortus (see table 6-2). Br. suis and Br. abortus cannot be separated by this method. The brucellae show differential sensitivity to a number of aniline dyes, for example, thionine, basic fuchsin, and crystal violet. Carbohydrates are fermented by the three species in the following manner. Brucella melitensis ferments only glucose while Br. abortus ferments glucose, inositol, mannose, and rhamnose. Brucella suis ferments glucose, mannose, maltose and trehalose. Brucella suis hydrolyzes urea rapidly while the other two are very slow or do not hydrolyze at all. Nitrates are reduced by all three species and all have catalase. Brucella suis is very active and Br. abortus is least active for catalase. Hydrogen sulfide is produced abundantly by Br. suis, to a lesser extent by Br. abortus and only in trace amounts by Br. melitensis. Indole, methyl red, and Voges-Proskauer tests are all negative for all three species. Ammonia is produced in varying amounts. Brucella suis produces the greatest amount of urease, catalase, and nitrate reduction. The best test is to use aniline dyes and observe the growth or inhibition in them.

	CO_2 Requirement	H_2S Production	Growth with Thionine		Growth with Basic Fuchsin		Agglutination in mono-specific sera		Most common host reservoir
			a	b	c	d	A	M	
Br. melitensis	-	-	-	+	+	+	+(-)	+(-)	Sheep, goats
Br. abortus	+	+(-)	-(+)	+	+	+(-)	+(-)	±	Cattle
Br. suis	-	+(-)	+(-)	+(-)	V	V	+	-(+)	Pigs, hares, reindeer
Br. neotomae	-	+	-	-	-	-	+	-	Desert wood rat
Br. ovis	+	-	+	+	+	+	r	r	Sheep
Br. canis	Inhibited	-	+	+	-	-	r	r	Dogs

THIONINE CONCENTRATION
BASIC FUCHSIN CONCENTRATION: (a) 1:25,000, (b) 1:50,000, (c) 1:50,000, (d) 1:100,000; using tryptose agar

A = abortus; M = melitensis
+(-) = positive, rare negative; -(+) = negative, rare positive
V = variable
r = rough colony and can not be typed

Table 6-2. Differential characteristics of Brucella species.

Section III. HAEMOPHILUS

6-8. MORPHOLOGY

The genus Haemophilus is a heterogeneous group of small, gram-negative bacilli that are nonmotile and nonspore-forming. They are minute gram-negative rods that may be pleomorphic (occurring in various distinct forms), sometimes almost coccal, and sometimes threadlike. Although in many instances the-various stains *of* Haemophilus species are microscopically indistinguishable, the better known forms have been shown to exhibit some cellular features of differential value.

a. Haemophilus influenzae is usually observed as very small bacilli, rarely exceeding 1.5 microns in length and 0.3 microns in breadth. The cells occur predominantly as coccobacillary forms, although longer, slender bacilli may be observed. There is a marked tendency for the organism to produce long thin filaments, or other aberrant forms in culture. The organisms are more difficult to stain than most bacteria. In smears of young smooth (S) colonies, definite capsules are exhibited. These capsules are rapidly dissolved by autolytic enzymes as the culture becomes older.

b. Haemophilus haemolyticus strains vary from small rods resembling H. influenzae, to larger bacillary forms occurring as irregularly shaped filaments. In addition, many pleomorphic forms are often observed which may be spheroid, tenpin, or dumbbell-shaped, or even club-shaped in appearance.

c. Haemophilus ducreyi (Ducrey's bacillus) appears in gram-stained smears of tissue exudates as short ovoid rods in end-to-end pairs (diplobacilli) or short chains. Individual cells are about 1 to 1.5 microns in length and 0.6 microns in breadth.

6-9. CULTURAL CHARACTERISTICS

a. **Growth Requirements.** Haemophilus species grow best at 37º C in 10 percent CO_2.

(1) The genus is composed of hemophilic or hemoglobinophilic species. Haemophilus influenzae and closely related species require two factors in blood for growth. One factor, designated as X, is a heat-stable derivative of hemoglobin. The other factor, designated as V, is a heat-labile fraction of blood consisting of coenzyme I. The V factor is also formed by yeasts, staphylococci, and certain other microorganisms. Blood or commercially available additives should be incorporated in isolation media for the Haemophilus species requiring X and/or V factors.

(2) Some variations exist in the ability of certain haemophilus species to grow on human blood. The use of human blood as the enrichment is not recommended; it should not be used since it may contain antibodies against this organism. Rabbit blood is the blood of choice.

(3) Sheep blood contains substances that inhibit the growth of Haemophilus species, and can only be used for the preparation of chocolate agar. The inhibitory substances in chocolate agar are destroyed by the necessary temperature (70º C to 75º C) required of the sterilized agar base for lysis of red blood cells. Since heat-labile V factor is also destroyed in preparing chocolate agar, it must be restored to the medium. This is accomplished by adding enrichment supplements A or B to chocolate agar at a temperature of 50º C.

(4) If staphylococci are able to grow on a blood agar medium, it may be heavily inoculated with the specimen presumed to contain a Haemophilus species. Immediately thereafter, one or two streaks of staphylococcal organisms are applied at right angles to the primary streaks. After 24 to 48 hours of incubation, Haemophilus influenzae or similar species requiring V factor will appear as small colonies growing in close proximity to the colonies of staphylococci. This is referred to as "satellitism" and results from the production of V factor by the Staphylococcus species. Diffusion of V factor into the surrounding medium provides a readily available enrichment source for the Haemophilus species, and little or no growth of the organism will occur except along the staphylococcal growth line.

b. **Observable Colony Characteristics.** Haemophilus influenzae strains isolated from most pathologic sources occur in the so-called S (smooth) phase. The S colonies are raised, slimy, mucoid confluent, and nonhemolytic. Microscopic examination will reveal the presence of capsules. The R (rough) colonies are small, nonhemolytic, transparent to translucent, and present a discrete dewdrop appearance. The cells of the R phase are nonencapsulated. The S phase colonies are readily differentiated from R phase colonies in that S colonies are larger (2 to 4 mm) and more slimy and irregular than the well-defined colonies of the R phase. After 2 to 3 transfers on culture media, the encapsulated S colonies revert to the nonencapsulated R phase. The colonies of H. haemolyticus may be confused with the colonies of beta streptococci because of similarity of gross morphology and hemolysis. All colonies appearing to be beta streptococci must be verified by gram staining.

6-10. PATHOGENICITY AND IDENTIFICATION

Characteristics useful in differentiating the Haemophilus species are given in table 6-3.

ORGANISM	NEED FOR X FACTOR	NEED FOR V FACTOR	HEMOLYSIS
H. Influenzae	+	+	-
H. duereyi	+	-	Slight
H. aegyptius	+	+	-
H. parainfluenzae	-	+	-
H. haemolyticus	+	+	+
H. parahaemolyticus	+	+	-
H. aprophilus	+	-	-

Table 6-3. Hemolytic activity and growth requirements of *haemophilus species*.

a. **Haemophilus influenzae.** The encapsulated strain of H. influenza may cause pharyngitis, conjunctivitis, otitis, sinusitis, pneumonitis, or meningitis. Meningitis is rare, occurring primarily in children under three years of age. The nonencapsulated variety of H. influenzae is considered to be normal flora in the upper respiratory tract of adults. Haemophilus influenzae is a fastidious organism and requires a medium enriched with blood or hemoglobin to supply the X factor and also a supplement for the V factor. All strains of H. influenzae reduce nitrates and are soluble in sodium deoxycholate; indole is produced by the encapsulated strains and fermentation reactions are variable. In cases of suspected meningitis caused by H. influenzae, spinal fluid is submitted. The specimen should be centrifuged and the supernatant disposed of in accordance with local laboratory procedures. The sediment is inoculated on a blood agar and a chocolate agar plate to which supplement has been added, or on which a staphylococcal streak is make. A smear is also made, gram-stained, and thoroughly examined. If gram-negative coccobacilli are seen, the requesting physician should be immediately notified. Throat swabs may also be submitted, and if H. influenzae is suspected, a chocolate agar plate should be inoculated along with a blood agar plate. Satellitism will occur if bacteria producing the V factor are present. R phase colonies of H influenzae are usually seen in throat swabs of carriers and adults, where the organism is normally present in small numbers. When H. influenzae is recovered from throat swabs, its presence should be confirmed by subculturing to chocolate agar with added supplement or with a "staph" streak. Final identification of H. influenzae is accomplished by serotyping.

b. **Haemophilus ducreyi**. Haemophilus ducreyi is the etiologic agent of chancroid, a venereal disease characterized by the formation of ragged, soft ulcers in the genital region. The regional lymph nodes of the groin may become secondarily infected and are referred to as buboes. The ulcers differ from typical hard chancre of primary syphilitic lesions in that their edges are soft. Such localized infections are called soft chancres or chancroids. Chancroid ulcers often become infected with other bacteria. In gram-stained exudates from chancroids, these gram-negative organisms, about 1.3 microns by 0.5 microns in size, may appear singly, in small clusters, or in parallel rows suggestive of a school of fish. H. ducreyi is extremely hard to culture. When infections are considered to originate from H. ducreyi, material collected from beneath the craterlike edges of soft chancres (chancroids) are examined. Specimens may also be obtained by aspiration of any intact buboes that may be present.

c. **Haemophilus aegyptius**. Haemophilus aegyptius (Koch-Weeks bacillus) causes a highly communicable form of conjunctivitis ("pink eye") and morphologically resembles H. influenzae. It requires both the X factor and the V factor for growth and will produce satellite colonies when grown with adjoining Staphylococcus colonies.

Section IV. BORDETELLA

6-11. INTRODUCTION

The genus Bordetella consists of three species, Bordetella pertussis, Bordetella parapertussis, and Bordetella bronchiseptica, which are all minute, gram-negative coccobacilli and very closely resemble Haemophilus species. Bordetella pertussis is the causative agent of whooping cough. The Bordetella species do not require the X factor or the V factor for growth; however, blood is definitely a stimulus to members of this group and should be incorporated in isolation media. The glycerin-potato-blood agar of Bordet and Gengou is most often used for cultivation of these organisms. The growth of Bordetella pertussis on this medium is slow, usually requiring from 3 to 5 days' incubation. The colonies are small and dome-shaped, possessing a gray metallic luster, resembling mercury droplets. Although the colonies of pertussis bacilli are beta hemolytic, the zone of hemolysis is difficult to observe since Bordet-Gengou agar contains 15 to 20 percent blood.

6-12. IDENTIFICATION

Species identity is confirmed by the use of FA (fluorescent antibody) staining, slide agglutination tests with absorbed sera, or biochemical tests.

a. **Biochemical Reactions.** The relative inertness of Bordetella cells, is indicated by their FAILURE to ferment sugars, liquefy gelatin, produce indole, produce acetyl methylcarbinol, or produce H_2S. However, most strains are catalase-positive, litmus milk is alkalinized, and most media are alkalinized. B. bronchiseptica rapidly produces urease. Other differential traits are illustrated in table 6-4.

b. **Slide Agglutination Tests.** Specific antisera may be used to identify Bordetella organisms as soon as growth is apparent on Bordet-Gengou plates.

TEST	B. pertussis	B. parapertussis	B.bronchiseptica
Urease	-	+	+4h
Nitrate	-	-	+
Motility	-	-	+
Growth on blood-free peptone agar	-	+	+
Browning of peptone agar	-	+	+

Table 6-4. Differential characteristics of Bordetella species.

Section V. FRANCISELLA TULARENSIS

6-13. MORPHOLOGY

Francisella tularensis (formerly known as Pasteurella tularensis), the gram-negative coccobacillus that causes tularemia, is much smaller than the plague bacillus; it is 0.3 to 0.5 by 0.2 microns upon initial isolations built it becomes rodlike upon transfer. It may be quite pleomorphic and filamentous in old cultures. It occurs singly, is nonencapsulated in vitro and nonmotiles stains faintly with aniline dyes, and may show bipolar staining. Capsulated forms occur in vivo.

6-14. CULTURAL CHARACTERI.STICS

F. tularensis will not grow on ordinary media and may fail to grow even on highly enriched blood agar unless cystine or cysteine is added. Cultivation is most successful when using blood-cystine-glucose agar slants. Primary growth of F. tularensis from specimens usually requires 4 to 7 days' incubation when blood-cystine-glucose agar slants are used. In young cultures the colonies are very thin, although gram-stained smears of fluid from the base of the slant will reveal numerous cells. Later, relatively heavy growth of small, gray, transparent to translucent, mucoid colonies develop. Subcultures to blood-cystine-glucose agar usually yield abundant growth within 2 to 3 days.

6-15. LABORATORY IDENTIFICATION

F. tularensis is an extremely dangerous pathogen and strict aseptic technique must be maintained at all times. When tularemia is suspected, special media must be inoculated in addition to the routine media--blood agar and thioglycollate broth. This is necessary since other bacterial agents may cause systemic disease similar to tularemia. Direct microscopic examination of F. tularensis is seldom productive. The final identification of colonies suspected of being F. tularensis is usually made by testing for agglutination with a standard, specific antiserum and the demonstration of virulence by intraperitoneal inoculation of guinea pigs. Inoculation of animals is a highly dangerous procedure that requires special facilities.

6-16. PATHOGENICITY

F. tularensis is the causative agent of tularemia; which is primarily a disease of rodents, rabbits, hares, and birds. Humans are only accidental, terminal hosts. The reservoir of infective agents is maintained among wild animals by biting flies (chrysops), ticks, and the rabbit louse; all of which are capable of spreading the disease from animal to animal. Humans can contact tularemia either directly through handling the flesh of infected animals or indirectly by an insect vector. The primary source of human infection is the rabbit. In the process of preparing the animal for food, the bacilli may enter through cutaneous abrasions or possibly through the intact skin. Aerosols of body fluids from infected animals may result in infections of the conjunctivae or lungs. Following invasion of the skin and mucous membranes, an ulcerating papule usually develops at the site of entry. The bacilli spread rapidly to the regional lymph nodes that become enlarged and suppurative. A transient bacteremia during the first week of illness serves to distribute the organisms to various internal organs where various foci of infection develop. As the disease progresses, pneumonia and fulminating septicemia may develop, resulting in death in untreated cases. Frequently the clinical signs are suggestive of the portal of entry. This is evidenced in that infections may be oculoglandular, following infection by way of the conjunctivae; ulceroglandular, following entry through the skin, or pneumonic, resulting from primary inhalation of infectious droplets. In some cases, there are no signs of localized 'involvement, but only the picture of a febrile systemic illness.

Section VI. MISCELLANEOUS ORGANISMS

6-17. BACTEROIDES FRAGILIS

Bacteroides fragilis is a nonsporogenous, nonencapsulated, nonmotile, gram-negative bacillus. It is a strict anaerobe requiring the complete absence of atmospheric oxygen. The optimum temperature for growth and isolation is 37º C. Initial isolation of this organism is accomplished by using Brewer's thioglycollate broth or deep tubes of meat media overlayed with paraffin. This will provide the anaerobic conditions necessary for growth of this bacterium according to the anaerobic techniques in Lesson 2, Section II. The organisms are transferred from this media to blood agar plates or ascitic agar plates to obtain pure colonies of Bacteroides fragilis. The colonies when grown in pure culture are smooth, convex, with an entire (even) edge, and they may exhibit pigmentation. The pigment may be white, gray, or yellow. The organism may be found in specimens taken from the mouth, vagina, or the intestinal tract. In fact, it is the anaerobe most frequently isolated from clinical infections, and it is the most common organism in the normal intestinal tract.

6-18. STREPTOBACILLUS MONILIFORMIS

Streptobacillus moniliforrnis is a gram-negative bacillus. The bacilli may be small and slender or may be looped or have curved filaments. The loops resemble a necklace or a string of beads (moniliform). Initial isolation is best obtained by using thioglycollate broth with the addition of ascitic fluid. Further culture may be obtained in solid media such as blood agar or serum agar with blood. The colonies growing on these media are small, smooth, and glistening, with irregular edges, and are colorless to gray. This organism is a normal inhabitant of the throat and nasopharynx of the rat. The infection in man is also known as rat-bite fever and is contracted by the bite of this animal.

Section VII. ANTIBIOTIC SENSITIVITY TESTS

6-19. GENERAL COMMENTS

a. Chemotherapy involves the systemic use of chemical agents for the treatment of infectious disease, and antibiotics are substances, having antimicrobial properties. The basis of chemotherapy, then is selective toxicity; the drug will destroy the disease-producing microbe nonmotile having relatively little effect on the host. This selective toxicity represents the main functional difference between antibiotics and disinfectants. Disinfectants and antiseptics lack cellular specificity.

b. An antibiotic must be effective in vivo in concentrations not harmful to the host. It is possible for an antibiotic to be effective in vitro ("in a test tube") but not in vivo. In vitro the antibiotic is in contact with only one biological system, namely that of the bacteria. In vivo an entirely different situation exists. In vivo the antibiotic is in contact with at least two biological systems, the bacteria and the host. The response of the host may be such that it alters the antibiotic to a form not effective against the pathogenic bacteria. The in vivo activity of the antibiotic is extremely complex and the mechanism of antibiotic activity in vivo is not generally known.

c. The laboratory specialist should take great care in performing antibiotic sensitivity tests and reporting their results. He should try to attain a high lever of accuracy and dependability. The laboratory report is in effect a recommendation for the usefulness or withdrawal of an antibiotic. In either case, the consequences for the patient can be tremendous.

6-20. ANTIBIOTIC RESISTANCE

Of extreme importance in the area of chemotherapy is the subject of bacterial resistance to antimicrobial agents. In the bacteriology section it is often necessary to determine an effective agent for an organism that has become resistant to another more commonly used agent.

a. **Resistance by Mutation.** An organism becomes resistant by mutation. Within a given bacterial population, some bacteria will develop that are more resistant to an antibiotic than the rest of the population due to the natural occurrence of mutations. The administration of an appropriate antibiotic kills all of the population except the resistant mutants. These mutants are called first-generation mutants. Each succeeding generation of the first generation mutants exposed to the antibiotic will demonstrate a greater degree of resistance to the agent. Most drug resistant bacteria are "selected" in this manner. An example of this method of resistance having great clinical significance is the case of penicillin-resistant Staphylococcus aureus. When penicillin was first used, it was employed indiscriminately. In effect, first-generation mutants were selected out from the general bacterial population. Through the years, resistance to penicillin increased through all succeeding generations of this bacterium. Today, the penicillin-resistant "staph" often presents a grave problem.

b. **Cross-Resistance.** Second, bacteria may become resistant to an antibiotic by a process of cross-resistance. The organism develops resistance to another antibiotic that is similar chemically, even though the bacteria had not been exposed to the second agent. For example, bacteria resistant to streptomycin may also be resistant to dihydrostreptomycin, which is similar chemically.

6-21. PREVENTING EMERGENCE OF RESISTANT STRAINS OF BACTERIA

Once the problem of drug-resistant strains of bacteria was recognized, several ways of handling the situation were developed. First, high blood levels of the antibiotic must be maintained. This will help prevent resistance by holding back the emergence of first-step mutants. Second, combined therapy is sometimes very effective. Combined therapy involves the concomitant use of two or more antimicrobial agents to combat infectious disease. Under combined therapy, one drug may be effective against those mutants resistant to the other drug. Naturally, drugs for which the bacteria may be cross-resistant must not be used.

6-22. LABORATORY ANTIBIOTICS METHODS FOR DETERMINING SENSITIVITY OF AN ORGANISM TO ANTIBIOTICS

a. **Paper Disk Method.** A paper disk containing a predetermined concentration of antibiotic is placed on the surface of an agar plate previously inoculated with the organism to be tested. The size of a zone of inhibited growth surrounding the disk is used as an index of sensitivity. The size of a zone surrounding a disk is dependent upon such factors as the amount of inoculum, thickness of the medium, and diffusion and solubility of the antibiotics. The detection of isolated colonies growing within a zone of inhibition indicates the presence of resistant organisms from the culture. The disk method of antibiotic sensitivity is a convenient and rapid technique for use in the clinical laboratory. This method is discussed further later in this lesson.

b. **Tube Dilution Method.** Employing the serial dilution technique, decreasing concentrations of antibiotics are prepared in broth for inoculation with a culture of the organism to be tested. After inoculation and incubation of the dilutions with the organism, its sensitivity is determined by the presence or absence of growth in the varying concentrations of therapeutic agents. If there is no growth in the control tube, which contains no antibiotic, the test should be considered invalid. If there is excellent growth in the control tube, look for the tube containing the lowest concentration of antibiotic in which growth is inhibited. If there is a clear-cut distinction between two adjacent tubes in increasing antibiotic concentration, report the result as the lowest concentration of antibiotic in which' bacterial growth is inhibited. If there is a gradual decrease in the amount of growth as the concentration of antibiotic increases, report the results as the lowest concentration of antibiotic to which the bacterial growth is sensitive and the lowest concentration of antibiotic to which the growth is completely resistant. The tube dilution method is applicable to research procedures and other situations requiring quantitative determinations.

6-23. KIRBY-BAUER METHOD FOR DETERMINING BACTERIAL SENSITIVITY

a. A few well-isolated colonies (3 to 8) of the organism to be tested are transferred with a wire loop from the original culture plate to a test tube containing 4 ml of tryptase phosphate or Trypticase soy broth. (Both of these media will support the growth of the great majority of bacteria found in clinical infections.)

b. Incubate tubes two to five hours to produce a bacterial suspension of moderate cloudiness.

c. Dilute suspension, if necessary, with sterile water or saline solution to a turbidity visually comparable to that of a standard prepared by adding 0.5 ml of one percent barium chloride to 99.5 ml of one percent sulfuric acid (0.36N). An alternate procedure is to dilute overnight broth cultures to the density of the opacity standard (10- to 100-fold).

d. For sensitivity plates, large (15 cm) petri dishes are used with Mueller-Hinton agar (5 to 6 nm in depth).

(1) The large petri dishes are spacious enough to accommodate about nine disks in an outer ring and three or four more in the center.

(2) It is advantageous to place antibiotics that diffuse well in the outer circle and disks that produce smaller inhibition zones (such as vancomycin, polymyxin B, and Kanamycin) in the central area of the plate.

e. Plates are dried for about 30 minutes before inoculation and are used within four days of preparation.

f. The bacterial broth suspension is streaked evenly in three planes onto the surface of the medium with a cotton swab (not a wire loop or glass rod). Surplus suspension is removed from the swab by being rotated against the side of the tube before the plates are seeded.

g. After the inoculum has dried (three to five minutes) the disks are placed on the agar with flamed forceps or a disk applicator and gently pressed down to insure contact.

h. Plates are incubated immediately, or within 30 minutes. Incubate overnight (optimum 14 hours) at 37º C.

i. Measure zone diameters (including the 6-mm disk). A reading of 6 mm indicates no zone. Zone diameters may be read after incubation for 6 to 8 hours if they are needed.

j. The end point is taken as complete inhibition of growth as determined by the naked eye. See table 6-5 for interpretation of zone sites.

(1) In the case of sulfonamides, organisms must grow through several generations before inhibition takes effect. Slight growth (80 percent or more inhibition) with sulfonamides is therefore disregarded; the margin of heavy growth is read to determine the zone size.

(2) Swarming (spreading) of Proteus species is not inhibited by all antibiotics; a veil of swarming into an inhibition zone should also be ignored.

(3) If colonies are seen within a zone of inhibition, the strain should be checked for purity and retested.

k. Standard control organisms of known susceptibility should be employed at least once a week as a check on the activity of the disks and on the reproducibility of the test.

Antibiotic or Chemotherapeutic Agent		Disk Potency	mm-Inhibition Zone Diameter to nearest mm		
			Resistant	Intermediate	Sensitive
Ampicillin	Gram-negative and enterococci	10 ug	11 or less	12 - 13	14 or more
	Staphylococci and highly penicillin sensitive organisms	10 ug	20 or less	21 - 28	29 or more
	Haemophilus	10 ug	19 or less	--	20 or more
Carbenicillin[2]	Proteus and E. coli	50 ug	17 or less	18 - 22	23 or more
	Pseudomonas aeruginosa	50 ug	12 or less	13 - 14	15 or more
Cephalothin[3]	staphylococci	30 ug	14 or less	15 - 17	18 or more
Chloramphenicol		30 ug	12 or less	13 - 17	18 or more
Clindamychin		2 ug	14 or less	15 - 16	17 or more
Erythromycin		15 ug	12 or less	14 - 17	13 or more
Gentamicin		10 ug	12 or less		13 or more
Kanamycin		30 ug	13 or less	14 - 17	18 or more
Methicillin[4]		5 ug	9 or less	10 - 13	14 or more
Neomycin		30 ug	12 or less	13 - 16	17 or more
Nitrofurantoin[5]		300 ug	14 or less	15 - 8	19 or more
Penicillin-G	Staphylococci	10 U	20 or less	21 - 28	29 or more
	Other organisms	10 U	11 or less	12 - 21	22 or more
Polymyxin-B		300 U	8 or less	9 - 11	12 or more
Streptomycin		10 ug	11 or less	12 - 14	15 or more
Sulfonamide[5]		300 ug	12 or less	13 - 16	17 or more
Sulfonamide-trimethoprim (19:1)[5]		25 ug	10 or less	11 - 15	16 or more
Tretracycline		30 ug	14 or less	15 - 18	19 or more
Vancomycin		30 ug	9 or less	10 - 11	12 or more

[1]Class disk for ampicillin and hetacillin.
[2]A change to a 100-ug disk is being considered. This would entail new standards.
[3]Class disk for cephalothin, cephaloridine, cephalexin and cefazolin.
[4]Class disk for penicillinase-resistant penicillins with staphylococci.
[5]Urinary tract infections only.

Table 6-5. Kirby-Bauer susceptibility test method: zone size interpretative chart.

Continue with Exercises

EXERCISES, LESSON 6

INSTRUCTIONS: Answer the following exercises by marking the lettered responses that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided.

After you have completed all of the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. To determine the pattern of carbohydrate metabolism of oxidative bacteria, we use OF basal medium because it contains:

   a. Uric acid.

   b. Petrolatum.

   c. No peptone.

   d. Less peptone than other fermentation media.

2. When OF basal medium is inoculated with a fermentative organism, acidity generally occurs in:

   a. Only the open tube.

   b. Only the petrolatum-covered tube.

   c. Both the open and petrolatum-covered tube.

   d. Neither the open nor the petrolatum-covered tube.

3. Which of the following gram-negative bacilli causes serious infections in which blue-green pus may be produced?

   a. Escherichia coli.

   b. Bacteroides fragilis.

   c. Klebsiella pneumoniae.

   d. Pseudomonas aeruginosa.

   e. Proteus mirabilis.

4. Upon primary isolation, which of the following species of Brucella will not grow without increased carbon dioxide tension?

   a. suis.

   b. abortus.

   c. melitensis.

   d. canis.

5. Which of the following species of Brucella is pathogenic for man?

   a. All OF THE BELOW.

   b. abortus.

   c. melitensis.

   d. suis.

6. When examining blood cultures, the technician should be particularly cautious if the organism is suspected to be:

   a. Bacillus.

   b. Bordetella.

   c. Borrelia.

   d. Brucella.

7. Differentiation of the species of Brucella is based primarily on:

   a. The gram stain.

   b. Cellular morphology.

   c. Growth on Castaneda medium.

   d. Growth in presence of thionine and basic fuchsin.

8. The "satellitism" phenomenon is illustrated by:

   a. Staphylococcus, forming colonies in the presence of Haemophilus species.

   b. Streptococcus, species forming colonies in the presence of Haemophilus species.

   c. Haemophilus, species forming colonies in the presence of Staphylococcus.

   d. Haemophilus, species not being able to grow in the presence of Staphylococcus.

9. Haemophilus influenzae requires a medium containing:

   a. X factor only.

   b. V factor only.

   c. X and V factors.

   d. Neither X nor V factor.

10. Differentiation of the species of Haemophilus is based primarily on:

    a. Motility.

    b. Satellite growth.

    c. Gram staining reaction.

    d. Requirement for X and V factors for growth.

11. The causative agent of whooping cough belongs to the genus:

    a. Bordetella.

    b. Brucella.

    c. Haemophilus.

    d. Pasteurella.

12. Species identity of a Bordetella isolate is confirmed by:

    a. ANY OF THE BELOW.

    b. Biochemical tests.

    c. Slide agglutination tests.

    d. Fluorescent antibody staining.

13. Which of the following media is used for the cultivation of suspected Francisella tularensis?

    a. OF basal medium.

    b. Bordet-Gengou agar.

    c. Blood-cystine-glucose agar.

    d. Blood agar with added V factor.

14. Animal virulence studies for the presence of Francisella tularensis may be performed safely in most laboratories.

    a. True.

    b. False.

15. Which of the following is a strict anaerobe?

    a. Bordetella pertussis.

    b. Francisella tularensis.

    c. Bacteroides fragilis.

    d. Streptobacillus moniliformis.

16. The basis of chemotherapy is:

    a. Selective toxicity.

    b. General protoplasmic poisoning.

    c. Dermal application of disinfectants.

    d. Oral administration of antiseptics.

17. The results of antibiotic sensitivity tests are used primarily for:

    a. Epidemiology.

    b. Biological research.

    c. Pharmaceutical research.

    d. Determining how to treat a patient.

18. The diameter of a zone of inhibited growth around a paper disk containing an antibiotic is used as an index of the cultured organism's:

    a. Ability to synthesize the antibiotic.

    b. Ability to utilize the antibiotic as a nutrient.

    c. Sensitivity to the antibiotic.

    d. Virulence.

19. Using the tube dilution method for determining antibiotic sensitivity, the absence of bacterial growth in the control tube indicates:

    a. An invalid sensitivity text.

    b. A valid sensitivity test.

    c. A questionable sensitivity test.

    d. An organism extremely sensitive to the antibiotic being tested.

20. When the Kirby-Bauer method is used to test the sensitivity test of a staphyloccoccal strain to penicillin G, a zone size of 18 mm means that the staphylococci are:

    a. Resistant.

    b. Sensitive.

    c. Intermediate in sensitivity.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES, LESSON 6

1. d (para 6-1c(1))
2. c (para 6-1c(3))
3. d (para 6-2a)
4. b para 6-5b; Table 6-1)
5. a (para 6-6)
6. d (para 6-7a)
7. d (para 6-7b; Table 6-1)
8. c (para 6-9a(4))
9. c (para 6-10a; Table 6-2)
10. d (para 6-10a; Table 6-2)
11. a (para 6-11)
12. a (para 6-12)
13. c (para 6-14)
14. b (para 6-15)
15. c (para 6-17)
16. a (para 6-19a)
17. d (para 6-19c)
18. c (para 6-22a)
19. a (para 6-22b)
20. a (Table 6-5)

End of Lesson 6